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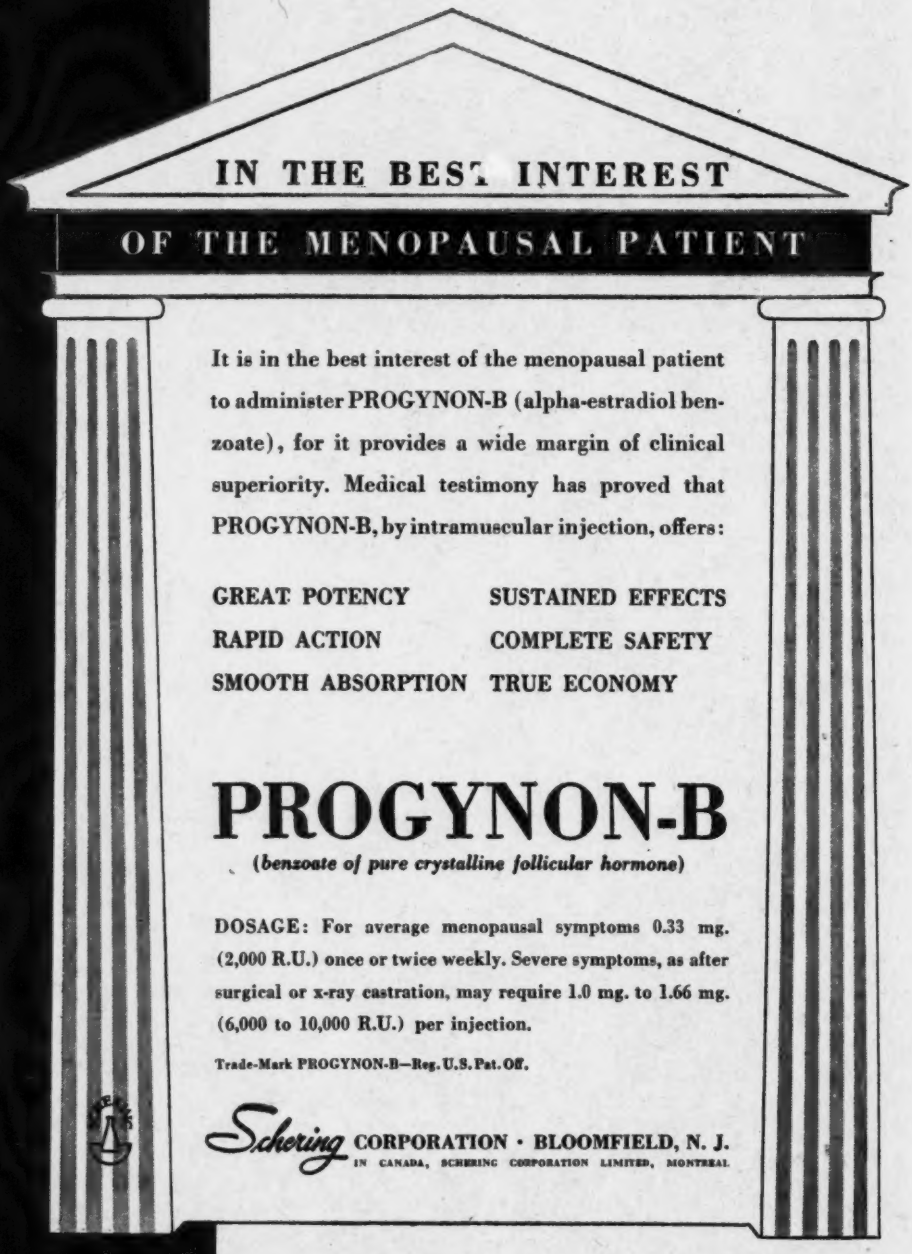
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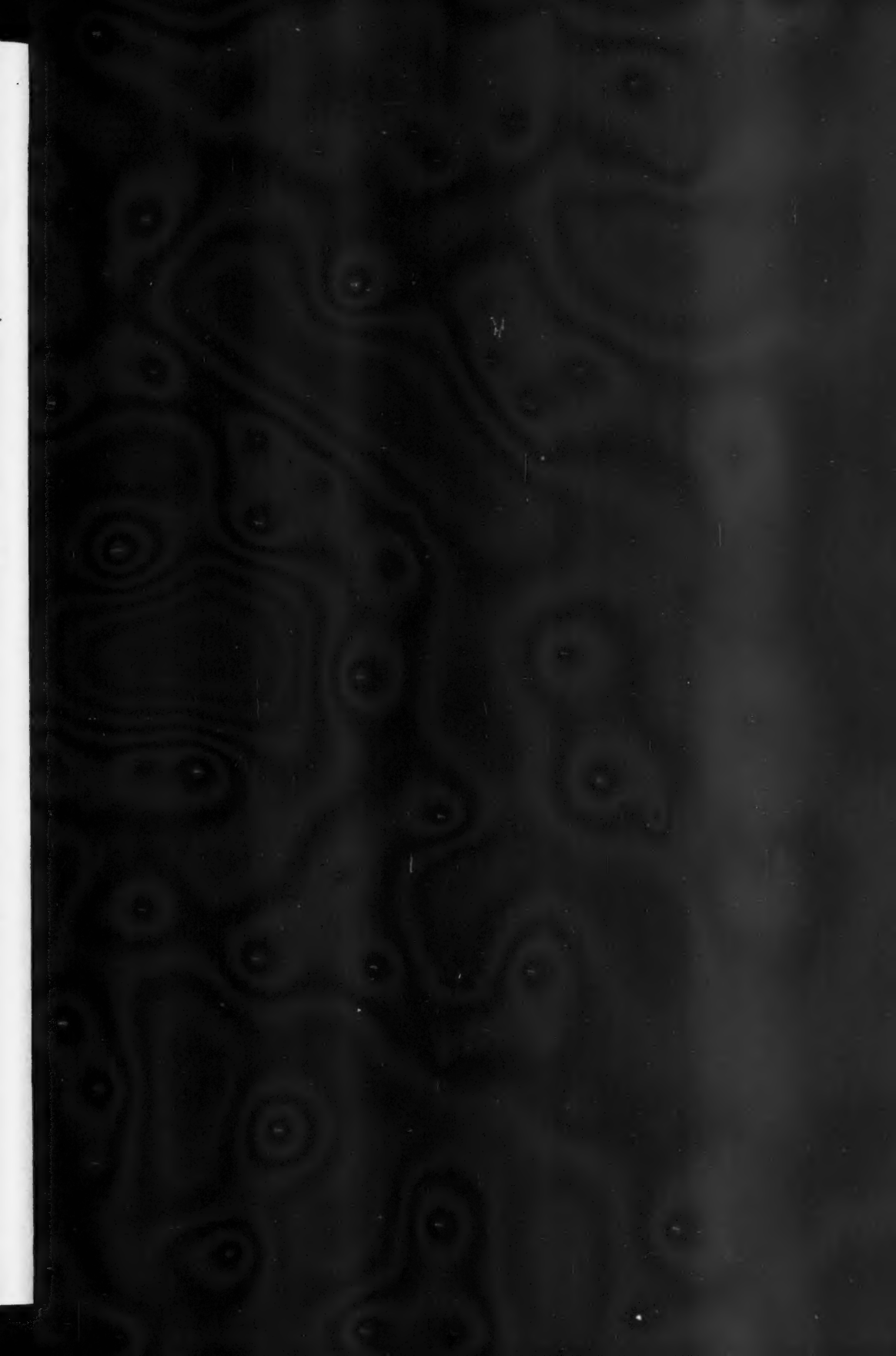

ENDOCRINOLOGY

VOLUME 39 OCTOBER, 1946 NUMBER 4

FACTORS AFFECTING SURVIVAL IN ADRENALECTOMIZED RATS[1]

ELAINE P. RALLI

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IN THE COURSE of studies on the alteration of melanin metabolism in adrenalectomized black rats maintained on diets deficient in the filtrate factors of Vitamin B (Ralli and Graef 1943, 1945), it was observed that survival after adrenalectomy was greatly prolonged when the diets were supplemented with a rice bran extract. The present report is concerned with the comparative effects of adrenal cortical extract, desoxy-corticosterone acetate, and calcium pantothenate, with or without 1 per cent sodium chloride solution, on the survival of adrenalectomized black rats.

Thirty-five day old black rats bred in the laboratories were placed on the filtrate factor deficient diet as previously reported (Ralli and Graef, 1943) and maintained on this diet for 30 to 35 days at which time the adrenal glands were extirpated. Sodium chloride was given as a 1 per cent solution in the drinking water. The rats were divided into two main groups, group 1 received the NaCl solution and group 2 received no salt at all after adrenalectomy. The subdivisions in each group were as follows: a control group receiving the deficient diet without any supplement: a group injected 6 days a week with 0.5 mgm. of DOCA (Roche Organon Co.): a group injected with adrenal cortical extract (Upjohn) 0.4 cc. 6 times weekly and a group whose diet was supplemented with calcium pantothenate. The calcium pantothenate was mixed into the diet, 40 mgms. per 100 gms. of diet. As the rats consumed from 10 to 15 gms. of the diet daily the intake of calcium pantothenate varied from 4 to 6 mgm. Each rat was kept in a separate cage and allowed to drink the 1 per cent NaCl solution ad lib. When measured amounts of the solution were given daily it was

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[1] This research was aided by a grant from the Josiah Macy Jr. Foundation.

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found that the rats drank from 25 to 35 cc. daily. In an additional sub group of Group 1 the concentration of the sodium chloride solution was increased to 2 per cent.

Tables 1 and 2 shows the effect of the experimental conditions on survival in the various groups. The survival is calculated on the per cent of the total number of rats adrenalectomized, surviving after varying periods. Of the 105 rats on 1 per cent NaCl and the deficient diet 50 per cent survived for 10 days, and 2 per cent survived 25 days. None survived at the end of 50 days and the average survival was 11.9 days ±5.4. When DOCA or adrenal cortical extract was injected

TABLE 1. ADRENALECTOMIZED RATS (GROUP I) RECEIVING 1% NaCl, FILTRATE FACTOR DEFICIENT DIET AND SUPPLEMENTS AS INDICATED

Experimental Conditions	No. of Rats	Per Cent of Total Surviving								
		10	25	50	75	100	150	200	250	300
		Days after Adrenalectomy								
Diet FFD +1% NaCl	105	% 50	% 2	% 0	%	%	%	%	%	%
Diet FFD +1% NaCl +DOCA	57	75	13	9	0					
Diet FFD +1% NaCl +Cortin	53	70	15	4	0					
Diet FFD +1% NaCl +Ca-pan.	70	100	91	81	67	62	50	30	17	7
Diet FFD +2% NaCl	26	38	0							

6 days a week, the survival after 10 days was almost identical for the two groups. Seventy-five per cent of the DOCA treated rats and 70 per cent of the rats receiving the cortical extract surviving in this period. In each of these groups a small number of rats survived 50 days but none survived for 75 days. When the diet was supplemented with calcium pantothenate (Walker Vitamin Co.) survival was definitely increased. All of the rats were alive after 10 days and 50 per cent survived 150 days. A few even survived for 300 days or longer, In order to check the completeness of adrenalectomy in the calcium pantothenate group, salt was withdrawn from 20 rats at intervals during the course of the experiment. All but 2 of the rats succumbed within a matter of days and these 2 died but survived for long enough periods so that the completeness of adrenalectomy might be questioned. Salt withdrawal in all adrenalectomized rats was associated with a striking weight loss and a decrease in food consumption. In the

group of rats that received a 2 per cent solution of NaCl and the deficient diet after adrenalectomy the average survival was 9.4 days ±5.0 and none of the rats survived for 25 days.

When the diet after adrenalectomy was not supplemented with 1 per cent NaCl (Table 2) survival was sharply curtailed in all groups. Here the average survival for each group is significant. On the deficient diet alone the survival was 6.2 days with an S.D. ±2.5; on cortical extract 5.3 days with an S.D. ±0.5, and on DOCA 11.5 days with an S.D. ±5.0. Without salt, calcium pantothenate was also ineffective, the average survival being 5.6 days ±1. As the rats on DOCA survived the longest time of any of the groups not receiving

TABLE 2. ADRENALECTOMIZED RATS RECEIVING THE FILTRATE FACTOR DEFICIENT DIET AND SUPPLEMENTS AS INDICATED BUT WITHOUT NaCl
Per Cent of Total Surviving

Experimental Conditions	No. of Rats	10 Days after adrenalectomy	25 Days after adrenalectomy	50 Days after adrenalectomy	75 Days after adrenalectomy	Average Survival in in Days
Diet FFD No NaCL	26	% 7	% 0	% 0	% 0	6.2±2.5
Diet FFD +DOCA	17	50	0	0	0	11.5±5.0
Diet FFD +Cortin	18	0	0	0	0	5.3±0.5
Diet FFD +Ca-pan	22	0	0	0	0	5.6±1.0
Diet FFD +DOCA +Ca-pan	21	62	0	0	0	12.5±4.5

salt, we studied an additional group in which DOCA was injected and the diet was supplemented with calcium pantothenate. Sixty-two per cent survived for 10 days, but none survived for 25 days and the average survival was 12.5 days with an S.D. ±4.5.

The weight changes in the different groups during the first 10 days after adrenalectomy are shown in Figure 1. The columns represent the difference between the average of the weight gained and the average of the weight lost in each group. The striking weight gain in the rats receiving 1 per cent NACl and calcium pantothenate is obvious. There was a consistent loss of weight in all the groups not receiving any sodium chloride after operation.

Chloride balance studies were done on 10 rats during periods of salt intake and salt deprivation.[2] The following animals were studied in

[2] The chloride balance studies are being continued by Dr. G. H. Stueck Jr.

this way: 2 unoperated rats on the deficient diet, 3 adrenalectomized rats on the deficient diet, and 5 adrenalectomized rats on the deficient diet supplemented with calcium pantothenate. As the results were

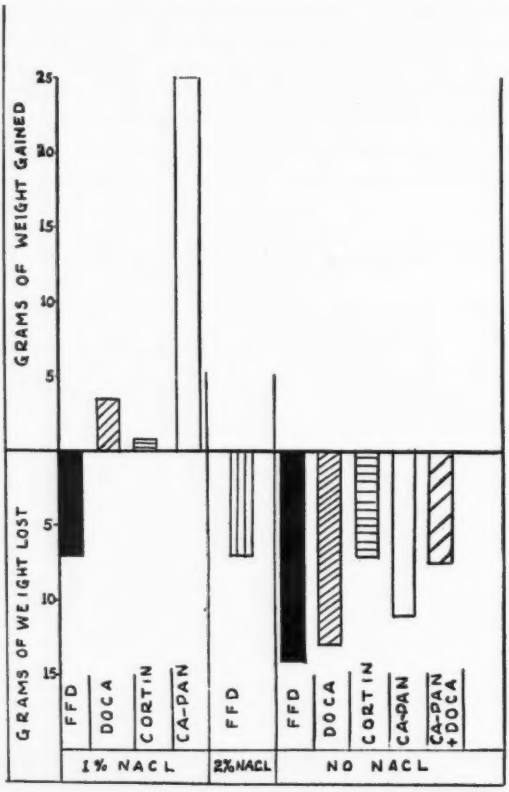

FIG. 1. The average weight changes in grams within the 10 day period following adrenalectomy in each group. Solid blocks represent rats on the filtrate factor deficient diet only; slanting cross hatch represents group receiving DOCA; straight cross hatch the group receiving adrenal cortical extract; wide slanting cross hatch a group receiving DOCA and calcium pantothenate; and the clear blocks represent the groups receiving the calcium pantothenate supplement to the diet. The results show that the rats receiving 1% NaCl in the drinking water lost less weight than those receiving no NaCl. The greatest gain of weight occurred in the rats receiving NaCl and calcium pantothenate. The rats receiving 2% NaCl and the deficient diet lost weight.

consistent an example of each is given in Figures 2, 3 and 4. The results are averaged for a 2 day period. The total intake of chloride is shown by the total column below the line, the hatched area represents the amount excreted and the clear area the amount retained. The

hatched area above the base line indicates the degree of negative balance.

During the period of sodium chloride administration the normal rats on the deficient diet (figure 2) were in positive chloride balance. When sodium chloride was withdrawn a negative chloride balance resulted and the amount of chloride excreted was quite constant for

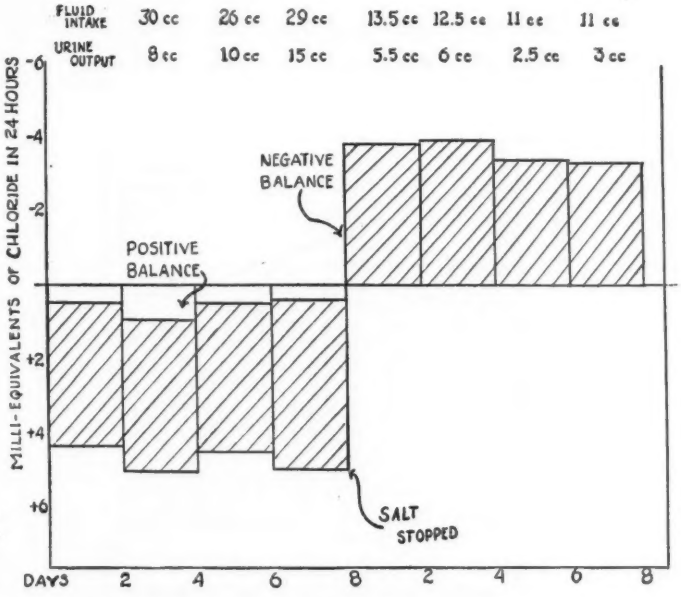

FIG. 2. Chloride balance studies on an unoperated rat maintained on the deficient diet. The figure illustrates a period when 1% NaCl was given and a period of salt deprivation. The total intake of chloride is shown by the length of the column below the base line, the hatched area represents the amount of chloride excreted, and the clear area the amount retained. The hatched area above the line indicates the degree of negative chloride balance following salt withdrawal.

the period of observation (20 days). During the period of salt deprivation the rats lost 1 to 2 grams of body weight. In the adrenalectomized rats receiving the deficient diet without calcium pantothenate (Figure 3) the deficient diet was begun when the animals were 38 days old and was continued for a longer period than usual prior to adrenalectomy. This was done in order that the animals' weights, at the time of the balance studies, would correspond more closely to those of the rats fed calcium pantothenate. During the period of chloride administration following adrenalectomy the rats were in positive chloride balance but when sodium chloride was withdrawn not only did a negative chloride balance result but none of the animals survived for more than

48 hours. In these adrenalectomized rats the period of salt deprivation was associated with a rapid loss of body weight, averaging 10 grams within the short period of survival following salt withdrawal. Of the 5 adrenalectomized rats receiving the deficient diet supplemented with calcium pantothenate and sodium chloride, 2 had been adrenalectomized for 335 days at the time salt was withdrawn and 3 had been

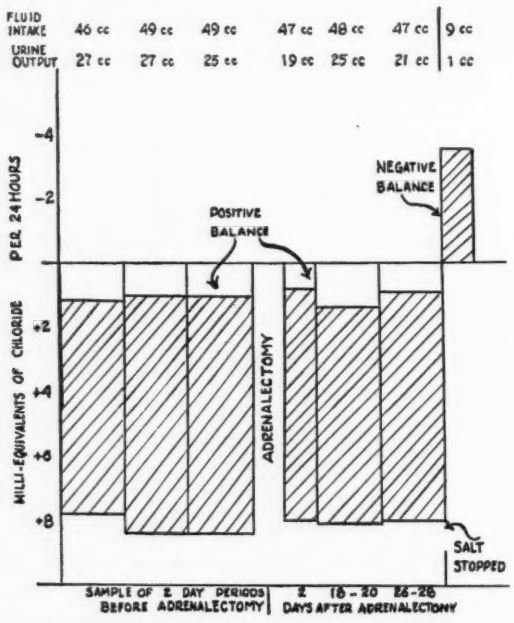

FIG. 3. Chloride balance studies on a rat fed the deficient diet for 55 days before adrenalectomy. The diet was not begun until the rat was 38 days old and was continued for a longer period than usual so that the animal's weight would correspond more closely to the weight of rats fed pantothenic acid. The figure shows the degree of chloride balance before and after adrenalectomy on the deficient diet plus 1% sodium chloride and then the effect of salt withdrawal on chloride excretion. The rat only lived long enough for one 24 hour chloride determination after salt deprivation.

adrenalectomized for 54 days when salt was withdrawn. Figure 4 illustrates the chloride balance studies on a rat that had been adrenalectomized for 335 days at the time salt was withdrawn. During the period of sodium chloride administration the animal was in positive chloride balance. Following salt withdrawal an unusual amount of chloride was excreted in the urine, e.g. 13.4 m. eq. in the first 24 hours. The animal survived salt withdrawal for 4 days. During the survival period he lost a total of 26 grams of body weight. The unusually high excretion of chloride during the period of salt deprivation

suggests that a considerable amount of salt had been stored in the tissue fluids during the period when the rat was receiving both calcium pantothenate and salt.

As can be seen in the figures salt withdrawal in the adrenalec-

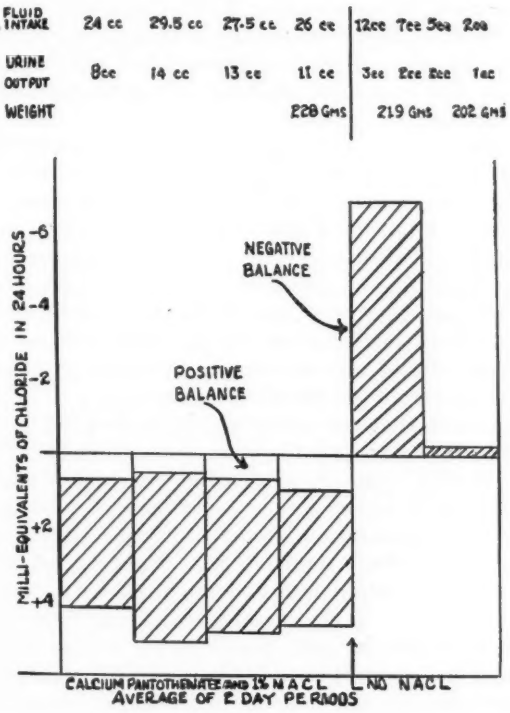

FIG. 4. Chloride balance studies on a rat adrenalectomized on 3–27–45 at 66 days of age. This rat was placed on the filtrate factor deficient diet at 35 days of age. After adrenalectomy the diet was supplemented with calcium pantothenate and 1% NaCl. After 335 days on this regime salt was stopped and the animal died in 4 days. Chloride balance studies were done on 2 day samples of urine for a period of 3 months and daily when salt was stopped. Although the amount of chloride excreted after salt deprivation is calculated on a two day period actually 13.37 M eq. were excreted in the first 24 hours and only .11 M eq. in the second 24 hours.

tomized rats was associated with a decrease in fluid intake and a decrease in urine output.

DISCUSSION

The results illustrate again the importance of sodium chloride for survival in adrenalectomized rats and show in addition that pantothenic acid is an essential constituent in the diet when the rat is

adrenalectomized. The significance of pantothenic acid is demonstrated by the fact that when the diet was supplemented with 1 per cent NaCl but was deficient in pantothenic acid survival was consistently limited. Furthermore this dietary deficiency conditioned the effectiveness of the adrenal cortical hormones as reflected by the length of survival. Supplementing the diet with calcium pantothenate and NaCl resulted in a remarkably long survival period. That the effect of pantothenic acid is closely associated with sodium chloride is suggested by the fact that without salt pantothenic acid was ineffective in prolonging survival.

The observations do not explain just what is the action of pantothenic acid. There are reports in the literature that suggest that pantothenic acid may be associated with both water balance and carbohydrate metabolism. Figge and Atkinson (1941) drew attention to the similarity of the symptoms of pantothenic acid deficiency in rats to the symptoms of dehydration. Gaunt, Lilling and Mushett (1946) noted that rats on a diet deficient in pantothenic acid developed water intoxication more readily than rats on normal diets. Administration of the adrenal cortical hormone or pantothenic acid protected the rats from the effects of excess water.

The suggestion that pantothenic acid is involved in carbohydrate metabolism has been reported by the work of Schaeffer et al. (1942). Observing growing dogs on diets deficient in pantothenic acid he found a lowering of blood glucose. Furthermore the deficiency was characterized by prostration and convulsions suggestive of hypoglycemia. Wright (1942) reported that the oral administration of glucose to fasting rabbits was accompanied by a lowering of the pantothenic acid level of the blood. He also interpreted this as evidence that pantothenic acid participates in glucose utilization. Elvehjem and his co-workers (1942) observed a decreased rate of pyruvate oxidation in the liver of pantothenic and biotin deficient rats. They suggest that these vitamins may be components of the enzyme systems concerned with the metabolism of pyruvate.

The action of pantothenic acid in increasing the survival of adrenalectomized rats receiving sodium chloride is still undetermined but the prolonged survival of the animals treated in this manner and the limitation of survival when the diet was deficient in pantothenic acid even with hormone therapy, are proof of its importance in the metabolism of the adrenalectomized rat.

SUMMARY

Experiments are reported in which black rats fed diets deficient in pantothenic acid were adrenalectomized. Following adrenalectomy one group of animals was given a 1 per cent NaCl solution and were either continued on the deficient diet alone or received the diet and injections of the adrenal cortical hormone or DOCA or were fed the

diet supplemented with calcium pantothenate. The second group of animals was treated in a similar manner, but *no sodium chloride* was given. Without sodium chloride survival, regardless of hormone administration or dietary supplement, was sharply curtailed. When 1 per cent sodium chloride was given the addition of calcium pantothenate to the diet resulted in a remarkably long period of survival, 50 per cent of the animals surviving for 150 days. The survival with calcium pantothenate and sodium chloride was longer than was obtained when the rats received hormone therapy plus sodium chloride and the deficient diet. The importance of sodium chloride to the survival of the pantothenic acid treated adrenalectomized rat was shown by the fact that withdrawal of salt resulted in death.

ACKNOWLEDGEMENTS

We are indebted to the Roche-Organon Co. of Nutley, N. J. for the desoxy-corticosterone acetate; to the Upjohn Co. of Kalamazoo, Mich. for the adrenal cortical extract and to the Walker Vitamin Co. of Mt. Vernon, N. Y. for the calcium pantothenate.

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EFFECT OF THYROID ACTIVITY UPON METABOLIC RESPONSE TO DINITRO-ORTHO-CRESOL[1]

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THIOURACIL, thiourea and similar compounds have been shown to lower the metabolism of experimental animals, probably by depressing the ability of the thyroid gland to elaborate its hormone (Franklin, Chaikoff and Lerner, 1944; Salter and McKay, 1944; Astwood, Bissell and Hughes, 1945). However, the animal rendered hypothyroid by these drugs still is able to give an essentially normal calorigenic response to thyroxin or desiccated thyroid substance (Dempsey and Astwood, 1943; Reineke, Mixner and Turner, 1945; Meyer and Ransom, 1945; Barker, 1945). It was thought of value to study the effect of dinitro-ortho-cresol (DNC) in thiouracil-treated rats, inasmuch as the dinitrophenols have been considered to exert their metabolism-stimulating effects directly upon the peripheral tissues (Magne, Mayer and Plantefol, 1932; Tainter and Cutting, 1933).

Although Tainter and Cutting (1933) stated that thyroidectomized rats gave as good a response as normals to 2,4-dinitrophenol (DNP), later reports have not been as conclusive. Dodds and Robertson (1933) were able to elevate the BMR of myxedematous patients with DNC, but the response to a single dose of drug was less marked than that of the normal individual. Dunlop (1934) did not observe any alteration in the calorigenic effect of DNP in a case of myxedema. Glaubach and Pick (1934) pointed out the implication in a previous report (Poole and Haining, 1934) of death following DNP ingestion that the toxicity of the drug had been increased by hyperthyroidism. Glaubach and Pick found that the hyperthermia induced in rabbits by a standard dose of DNP was heightened several times by previously treating the animals with thyroxin.

During research on the response of pigeons to calorigenic agents, Riddle and Smith (1935) found that DNP injected into a thyroidectomized pigeon produced a rise in oxygen consumption of only 50 per cent as compared with 178 to 320 per cent in normal pigeons. Alwall (1936) reported that, at several dose levels of DNP, thyroidectomized rabbits gave a much reduced elevation of metabolism and

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[1] The author wishes to acknowledge the assistance rendered during some of this work by A. W. Richardson.

hyperthyroid animals exhibited an enhanced response compared to the effect of the drug in normal rabbits.

The data of Leblond and Hoff (1944), obtained in a study of cardiac acceleration by various agents causing increased metabolism, also show a diminished response to dinitrophenol in the absence of the thyroid gland. In contrast to the acceleration of tadpole metamorphosis produced by thyroxin, DNP exerted no effect on this process (Cutting and Tainter, 1933). Cahane (1937) presented evidence to show that DNP caused less disappearance of muscle glycogen in thyroidectomized than in unoperated rabbits. There was no difference with regard to liver glycogenolysis.

METHODS

Adult albino rats, of the Sprague-Dawley strain raised in this laboratory, were accustomed to the metabolism apparatus, and were trained to eat their daily food requirement in 5 hours, so that metabolism determinations were performed 16 hours post-prandial (Barker, 1945).

DNC was injected intraperitoneally as the sodium salt, in doses of 5 or 10 mgm. of the free alcohol per kilo body weight. Animals were rendered hypothyroid by the inclusion of 200 mg. of thiouracil[2] per 100 gm. of diet (powdered Purina Laboratory Chow). Desiccated thyroid substance[3] was administered by stomach tube as a 5 per cent suspension in water. The dose used was 50 mg. per 100 gm. body weight per day, usually for 4 days prior to the day of the experiment.

The Benedict (1930) multiple-chamber closed-circuit apparatus was employed. The amount of oxygen consumed was measured in several 30 minute periods following an equilibration period of 20 or 30 minutes. Following the injection of the DNC and equilibration, the metabolic rate was calculated from the first two 30 minute periods, since these were found to differ less than 10 per cent with each other, and since succeeding periods fell off more rapidly.

RESULTS

Table 1 summarizes the data obtained on animals of both sexes, since no differences in response were encountered. When a dose of 10 mg. DNC per kg. body weight was injected into the control rats, it caused an increase of 37.8 cc. of oxygen above the basal level of 99.0 cc. oxygen per 100 gm. body weight per hour. This represents a 38.1 per cent elevation.

The metabolic rate of the animals was lowered to 80.3 cc. oxygen per 100 gm. per hour after they had been on the thiouracil-containing diet for about 3 weeks. At this time, injection of 10 mg. DNC per kg. caused an increase of only 10.8 cc. oxygen. This is less than one-third of the actual stimulation produced in the control animals, but

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[2] Deracil (thiouracil) was furnished through the courtesy of Dr. Stanton M. Hardy of the Lederle Laboratories.

[3] The desiccated thyroid was generously supplied by the Cudahy Packing Company.

amounts to 13.4 per cent because of the approximately 20 per cent depression in resting basal.

Several rats (8 in number) not shown in the table were thyroidectomized, which procedure lowered their oxygen consumption to 87.6 cc. per 100 gm. per hour. Following 10 mg. DNC per kg., this value rose 17.3 cc. (+19.7 per cent). These animals were then placed on thiouracil; after 3 weeks, their metabolic rates were still further lowered to the average of 78.4, being elevated only 8.1 cc. (+10.3 per cent) by 10 mg. DNC per kg.

Only one set of 4 hyperthyroid animals was given the larger dose of 10 mg. DNC per kg. body weight, since the effects obtained on these

TABLE 1. EFFECT OF DINITRO-ORTHO-CRESOL ON OXYGEN CONSUMPTION

Animals		D.N.C.	Oxygen Consumption[1]		
Condition	No.	mg./kg.	Control	D.N.C.	D.N.C. Increase
Normal	27	10	99.0± 5.5[2]	136.8± 8.1	+37.8 (+38.1%)
Thiouracil	40	10	80.3± 6.4	91.1± 9.1	+10.8 (+13.4%)
Hyperthyroid	4	10	157.8	289.0	+131.2 (+83.2%)
Normal	16	5	99.5± 3.1	115.2± 4.7	+15.7 (+15.8%)
Hyperthyroid	15	5	152.9±10.0	192.0±14.0	+39.1 (+25.6%)

[1] Oxygen consumption, cc. oxygen per 100 gm. body weight per hour.
[2] Standard deviation.

were so violent. The actual increase was 131.2 cc. of oxygen or 83.2 per cent above the already heightened basal of 157.8 cc. per 100 gm. per hour. These animals were seen to be in obviously acute distress, and two died shortly, one about 2 hours and the other about 3 hours after the injection.

Hence, a new series was set up, using one-half the amount of drug. This produced a metabolic stimulation in the normal rats of only 15.7 cc. of oxygen, 15.8 per cent above the basal of 99.5 oxygen per 100 gm. per hour. When the smaller dose of DNC was injected into the hyperthyroid animals, the oxygen consumption was elevated 39.1 cc., somewhat more than double the response of the controls. Because of the high basal, this increase was only 25.6 per cent.

DISCUSSION

The findings reported in this paper, gathered in Table 1, support the accumulating evidence that the activity of the thyroid gland is involved in some way in the metabolism-stimulating properties of the dinitrophenols. Animals rendered hypothyroid by thiouracil treatment showed a greatly reduced response to DNC as compared to controls. Hyperthyroidism produced by administration of desiccated thyroid substance considerably enhanced the animals' metabolic response to DNC.

Three possible modes of action may be considered briefly: first,

DNC acts through the thyroid gland; second, the thyroid hormone maintains certain tissue enzyme systems which are essential for the DNC effect; third, DNC acts through some other endocrine, such as the adrenal medulla, whose activity is highly dependent upon the thyroid (Barker, Fazikas and Himwich, 1936).

The first of these is improbable, considering the marked differences in time relations of the metabolic response to a dinitrophenol (a few minutes) and to thyrotrophin, known to stimulate the thyroid gland (many hours). However, Cahane (1937) reported that 10 mg. per kg. injected for several days into rabbits and guinea-pigs produced thyroid hyperplasia. This worker also quoted Parhon and Milcou as having obtained histological evidence of thyroid hyperfunction in two dogs after treatment with DNP for two months. No sections or other data were shown and the original of this latter paper was not available. Sanchez-Calvo (1940) claimed to have produced histological evidence of thyroid gland stimulation in guinea-pigs by intragastric administration of 10 mg. DNP per day for 5 days. No thyroid sections or metabolic data were given. The present author has been unable to detect any such effect in rats even following 14 days of 4 injections per day of 15 mg. DNP per kg. body weight.

Support for the concept that the dinitrophenols are unable to stimulate metabolism normally in hypothyroidism because of inadequate tissue enzyme activity was advanced by Alwall. He reported greatly decreased in vitro effects of DNP on minced tissues removed from hypothyroid rabbits. In this laboratory (Barker, 1946), tissues from hypothyroid rats have been found to respond to DNC in vitro at least as well as tissues from normal rats. Furthermore, there seems to be no enhanced in vitro effect in the case of tissues of hyperthyroid rats. These results were obtained using intact tissues; minced tissues have not been tried.

The third possibility, that of involvement of some other endocrine, has been investigated only with regard to the adrenals. No positive evidence for either adrenal medullary or cortical participation in DNC stimulation of metabolism has been obtained.

It is apparent, therefore, that the marked differences in response of hypothyroid, euthyroid and hyperthyroid animals cannot be explained at the present time.

SUMMARY

When dinitro-ortho-cresol was injected intraperitoneally into normal rats in a dose of 10 mg. per kg. body weight, the resting metabolic rate was increased 37.8 cc. oxygen per 100 gm. per hour over the basal of 99.0.

When the same amount of DNC was used with animals made hypothyroid with thiouracil, the oxygen consumption was increased only 10.8 cc. over the lowered basal of 80.3.

The response to 10 mg. per kg. was found to have increased to 131.2 cc. oxygen when hyperthyroid animals were employed. Because of the extremely marked effects, the dose was reduced to 5 mg. per kg. This amount raised the oxygen consumption of the hyperthyroid rats by 39.1 cc. in contrast to only 15.7 in the normals.

Thus, it can be concluded that the thyroid gland is involved in some manner in the metabolic response of the rat to the dinitrophenols. Various ways in which this relationship might be effected are considered.

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FURTHER STUDIES ON EFFECTS OF WATER SOLUBLE VITAMINS IN EXPERIMENTAL DIABETES[1]

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WITHIN a few weeks after withdrawal of yeast from their diet, depancreatized dogs receiving constant and adequate amounts of food, insulin, and pancreatin may develop glycosuria and lose nitrogen. In a preliminary attempt (Gaebler and Ciszewski, 1945) to relate this finding to known vitamins several of them were added at a time, for prolonged deficiency causes refusal of food and thus complicates experiments. Some results with individual vitamins can now be reported. In one animal which never responded like the others, an effort was made to exclude synthesis of vitamins by intestinal bacteria. We next determined whether or not glycosuria produced by slight insulin deficiency was influenced by the vitamin content of the food. Finally, we compared the amounts of glucose excreted during short fasts without insulin, after feeding complete and incomplete diets.

DIETS AND ANIMALS

Diet 1. This consisted of casein, 6800 gm.; cracker meal, 6800 gm.; corn oil, 4000 ml.; calcium phosphate, 900 gm.; salt mixture (Cowgill, 1923) 228 gm.; and pancreatin, 1530 gm. Haliver oil and water were added before feeding. A more deficient modification of this diet was made by substituting vitamin-free casein for the commercial grade and replacing cracker meal with four-fifths its weight of corn starch. Analyses of the modified diet agreed closely with those of the original one and indicated 37.4 per cent of protein, 29.5 per cent of starch, and 21.0 per cent of ether extract.

Diet 2. This mixture contained vitamin-free casein, Labco brand, 756 gm.; oleic acid, U.S.P., 334 gm.; sucrose, 553 gm.; glycerol, 37 gm.; calcium phosphate, $CaHPO_4 \cdot 2H_2O$, 48 gm.; and salt mixture (Phillips-Hart, 1935) 72 gm. Three drops of haliver oil were given daily. That pancreatin was not required for digestion of this mixture was an advantage. Since lipocaic preparations contain choline and inositol (Abels *et. al.*, 1943) these vitamins, and perhaps others, may be expected in pancreatin.

The experimental animals were 3 depancreatized bitches which had also been used in the preceding study.

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[1] The data in this paper are taken from a thesis presented by one of us (J.C.M.) to the Graduate Council of Wayne University in partial fulfillment of the requirements for the degree of Master of Science in the Department of Chemistry.

RESULTS

The experiments in Table 1 followed, after an interval of 9 days, those in the first 3 tables of the preceding paper (Gaebler and Ciszewski, 1945). They concluded 484 days of observations on the same animal.

During the first 3 periods shown in Table 1, this depancreatized dog, for the fifth time, developed glycosuria during vitamin deficiency, with food intake and insulin dosage constant. Slight losses of weight

TABLE 1. EFFECTS OF VITAMINS ON NITROGEN STORAGE AND GLYCOSURIA IN A DEPANCREATIZED BITCH RECEIVING A CONSTANT DOSE OF INSULIN

Period No.	Duration of period	Food intake per day	Supplement*	Weight change during period	Nitrogen stored per day	Glucose excreted per day
	days	*calories*		*gm.*	*gm.*	*gm.*
1	6	945	1, 2, 3	+110	−0.31	4.4
2	6	945	1, 2, 3	−110	−0.58	15.9
3	3	945	1, 2, 3	−110	−0.85	33.6
4	6	790	1, 2, 3, 4	−100	−1.40	30.8
5	6	945	1, 2, 3, 4	− 60	−0.11	30.2
6	9	945	1, 2, 3, 4, 5	+110	+0.23	18.9
7	12	945	1, 2, 3, 4, 5, 6	+110	+0.41	5.7
8	6	945	1, 2, 3, 4, 5, 6, 7	+220	+0.65	2.7
9	9	945	1, 2, 3, 4, 5, 6, 7, 8	0	+0.51	2.9
10	24	945	1, 2, 3, 5, 6, 7, 8	+110	+0.29	15.4
11	21	945	1, 2, 3, 4, 5, 6, 7, 8	0	+0.22	19.1
12	24	870	1, 2, 3, 4, 5, 6, 7	+220	−0.03	14.2
13	33	870	1, 2, 3	− 60	−0.10	8.0
14	21	870	none	− 50	−0.17	11.6
15	6	870	none†	0		6.4

* Numbers represent the following factors and daily doses:
1. Thiamine hydrochloride, 0.84 mg.
2. Riboflavin, 1.68 mg.
3. Nicotinic acid, 8.4 mg.
4. Inositol, 40 mg.
5. Pyridoxin hydrochloride, 2 mg.
6. Sodium pantothenate, 10 mg.
7. p-Aminobenzoic acid, 100 mg.
8. Ascorbic acid, 50 mg.

† Basal diet made more deficient by changing from diet 1 to modified diet 1.

Dog 33 was used. After the morning meal, 8 U. of protamine insulin and 3 U. of plain insulin were given separately. After the evening meal, 3 U. of plain insulin were given. All nitrogen balances are based on analyses of food, urine, and stools.

and nitrogen also occurred. Inositol, which had been omitted for 18 days, did not notably alter glucose output when added to the diet during period 4. Pyridoxin was added in period 6, after it had not been given for 87 days, and glucose excretion fell decidedly. Complete control of diabetes, as indicated by gain in weight, storage of nitrogen, and negligible glycosuria, began in period 7, when pantothenate was added again after having been omitted for 132 days.

We contemplated experiments in which one factor at a time would be removed from and returned to a diet containing all the factors studied, but were not successful. The mild glycosuria, which appeared

during period 10 (Table 1) when inositol was withdrawn, increased during period 11, when this vitamin was fed again. The animal, which originally weighed 14.0 kg., had been gaining weight steadily for over a year, was now quite fat, and weighed 16.5 kg. However, reduction of the calories in period 12 did not abolish glycosuria. The insulin dose, which was constant throughout the long series of experiments, apparently was no longer adequate. During periods 13 to 15, when the diet was deficient, glucose output was lower than it had been with

TABLE 2. EXPERIMENTS ON A DEPANCREATINIZED BITCH RECEIVING A PURIFIED DIET

Days of experiment	Weight at end of period	Average urine sugar	Dietary supplement
	kg.	*gm. per day*	
1–7	14.9	3.1	No supplement
8–14	14.9	5.6	No supplement
15–16	14.8		Some food refused
17–23	14.7	8.3	Thiamine, riboflavin, and nicotinic acid on and after the 17th day
24–30	14.5	14.0	
31–37	14.3	12.7	
38–44	14.3	11.4	Choline from 38th to 58th day
45–51	14.3	6.0	Pyridoxin and pantothenate on and after the 45th day
52–58	14.4	3.9	
59–65	14.2	9.1	Choline discontinued on 59th day
66–72	14.3	7.1	Inositol daily during this period

Dog 34, used in this experiment, received 195 gm. of diet 2, containing 952 calories, daily. When choline was fed, the dose was 500 mg. of the hydrochloride per day. Amounts of other vitamins are given in the footnote of table 1. Throughout the experiment the animal received 26 U. of insulin each day, 8 U. of protamine insulin and 5 U. of plain insulin, injected separately, after morning and evening meals.

the diet complete. In later experiments this was a common finding during insulin deficiency.

Experiments on another depancreatized dog, shown in Table 2, were begun immediately after a complete diet containing yeast had been replaced by the unsupplemented and purified diet 2. Within 2 weeks it was necessary to add thiamine, riboflavin, and niacin to assure complete food consumption. Glycosuria developed, and was little influenced by choline. Pantothenate and pyridoxin, now deficient for 44 days, were added simultaneously, since the appetite was again lagging. Glucose excretion returned to the original control value. Indefinite results of withdrawing choline for a week and substituting inositol are also recorded. Food consumption was constant throughout the experiment.

One depancreatized dog used in the present and preceding studies was able to maintain its weight for 2 or 3 weeks while receiving diet 1 unsupplemented. Anorexia was the only untoward effect. The deficiency glycosuria observed in the other two animals while they received thiamine, riboflavin, and niacin could not be demonstrated in

this animal even though 7 gm. of succinylsulfathiazole was added to each of the 2 meals daily for long periods. This is shown in Table 3. Since the dog still maintained its weight during 18 days of the third

TABLE 3. EXPERIMENT SHOWING THAT SUCCINYLSULFATHIAZOLE FAILED TO ALTER THE NEGATIVE RESULTS IN A REFRACTORY ANIMAL

Days of experiment	Weight at end of period	Average urine sugar	Substances added to the basal diet daily during each period
	kg.	*gm. per day*	
1–29	15.1	2.9	Thiamine, 0.84 mg., riboflavin, 1.68 mg., niacin, 8.4 mg.
30–62	15.3	3.0	The same, plus 14 gm. of succinylsulfathiazole daily
63–80	15.3	3.8	No vitamins, succinylsulfathiazole continued, 14 gm. daily

The animal, dog 35, received 97.5 gm. of diet 1 twice daily. 3 U. of plain, and 6 U. of protamine insulin were given separately after each of the two meals, a total of 18 U. daily.

TABLE 4. EFFECTS OF WATER SOLUBLE VITAMINS DURING INSULIN DEFICIENCY

Dog	Period	Number of days	Daily dose of insulin	Weight change during period	Glucose excreted per day	Dietary supplement*
			units	*kg.*	*gm.*	
34	1	33	22	−0.37	30.1	1, 2, 3
	2	18	22	−0.23	36.1	1, 2, 3, 4, 5, 6, 7
	3	23	22	−0.42	28.5	none
	4	12	22	−0.07	23.4	none†
	5	10	22	−0.27	28.9	yeast, 7.5 gm. daily
	6	4	25	+0.23	12.8	yeast, 7.5 gm. daily
	7	12	26	+0.07	10.3	yeast, 7.5 gm. daily
	8	13	26	+0.12	4.8	none
35	1	6	14	0.00	30.9	1, 2, 3
	2	17	14	−0.46	36.6	1, 2, 3, 4
	3	7	14	−0.07	41.4	1, 2, 3, 4, 5
	4	7	14	−0.16	34.5	1, 2, 3, 4, 5, 7
	5	5	14	−0.17	38.0	1, 2, 3, 4, 5, 6, 7
	6	10	14	0.00	36.1	1, 2, 3, 4, 5, 6, 7, 8
	7	8	18	+0.40	11.4	1, 2, 3, 4, 5, 6, 7, 8

* For significance of numbers see footnote, table 1.

† The basal diet was made more vitamin-free at this point by substituting modified diet 1 for diet 1. The caloric intake remained constant throughout both experiments. When yeast was fed, it replaced an equal amount of food.

period, when diet 1 was given unsupplemented, we continued the succinylsulfathiazole but used the more deficient modification of diet 1. During 4 days of this regimen, glucose excretion ceased altogether. The animal then refused its food. It has been suggested (Michaud *et al.*, 1944) that in the dog succinylsulfathiazole may not greatly reduce synthesis of vitamins by intestinal bacteria.

That one cannot mitigate the effect of insulin deficiency by improving the dietary supplement is shown in Table 4. During these experiments the caloric intake was adequate and constant. Each animal, while receiving about 4 U of insulin less than it required, lost weight and excreted large amounts of glucose until the insulin dose was increased. Detailed examination of data obtained while the insulin dose was constant shows that, in the case of dog 34, glucose output

TABLE 5. COMPARISONS OF GLUCOSE EXCRETION DURING SHORT FASTS WITHOUT INSULIN AFTER COMPLETE AND INCOMPLETE DIETS

Dog No.	Length of fast without insulin	Diet preceding fast	Glucose excreted		Other effects
			during fast	first day after fast	
	days		*gm.*	*gm.*	
33	3	deficient 27 days	115		Vomiting, collapse, death
34	3	complete 23 days	111		Severe vomiting, recovery
35	3	complete 23 days	138		Severe vomiting, recovery after long treatment
34	2	deficient 13 days	49	6	Condition good
34	2	complete 17 days	60	21	Condition good
35	2	deficient 29 days	35	7	Vomited bile, recovered
35	2	complete 17 days	45	56	Some vomiting
35	2	complete 49 days	45	48	Vomited hematin, recovered promptly

During periods when the diet is marked deficient, Dog 33 received diet 1 for 23 day and modified diet 1 for the 4 days just preceding the fast; Dog 34 received modified diet 1 for the entire 13 days; Dog 35 also received this diet for 29 days, but received 6 mg. of thiamine during this time. During all other periods the animals received diet 1, supplemented with 7.5 gm. of dried brewers' yeast per day. In the last experiment on Dog 35 a more complete salt mixture was used in diet 1 (Phillips-Hart, 1935).

rose in periods 2 and 5, when essential factors were added, and fell in periods 3 and 4, when the diet was made more deficient. The same relationship is seen in the case of dog 35, with only one exception—a fall in glucose output followed addition of para-aminobenzoic acid to the diet in period 4. Previous data on these 2 animals included 5 periods of 20 to 33 days each during which no experimental changes were made. When the average daily glucose excretion during the first and second half of each period was compared, the largest change was 6.8 gm., the average, 2.9 gm. The size of the changes in Table 4 is therefore not as convincing as the direction, which was almost always the reverse of the one we expected. This fact will be discussed later.

The effect of completeness or deficiency of the diet upon glucose output during a subsequent fast without insulin is shown in Table 5. The caloric intake and insulin dosage were adequate until both food and insulin were withdrawn for 2 or 3 days. Glucose excretion during,

or on the first day after these short fasts without insulin was largest when the diet preceding the fast was complete, probably because the stores of glycogen and protein convertible to glucose were greatest after complete diets. Although these depancreatized animals were maintained in apparently normal condition for 2 to 3 years, withdrawal of food and insulin for even 2 days caused severe vomiting. This well known finding was observed even when the diet preceding the fast contained yeast and a complete salt mixture.

DISCUSSION

It has been reported (Michaud *et. al.*, 1944) that young dogs grow normally and synthesize hemoglobin well while receiving purified diets supplemented with haliver oil and 6 water soluble vitamins: thiamine, riboflavin, niacin, choline, pantothenic acid, and pyridoxin. In the present study the same accessory factors, in adequate amounts, sufficed to stop loss of weight and glycosuria in a depancreatized dog receiving a purified diet and a constant dose of insulin (Table 2). There is evidence that dogs require other vitamins (Lambooy and Nasset, 1943; Smith, 1944; Krehl and Elvehjem, 1945) but a study of these would probably require the use of young and growing animals.

All of the water soluble vitamins required by the intact dog may influence nitrogen equilibrium in depancreatized dogs. Thiamine, riboflavin, and niacin, administered together in the previous study, largely prevented nitrogen loss, but other factors were required to produce positive balances. In the present study, separate addition of pyridoxin, pantothenate, and para-aminobenzoic acid (Table 1) was in each case followed by improvement in the nitrogen balance. In connection with control of glycosuria, evidence that all of these vitamins are involved is much less complete. Results with pantothenate and pyridoxin given separately (Table 1) and together (Table 2) clearly implicate these factors. Similar results were obtained in the earlier study when these two compounds were fed in combination with others. Concerning thiamine, riboflavin, niacin, inositol, and choline we know only that these factors together do not suffice to stop glycosuria (Tables 1 and 2).

There have been several instances in which susceptible animals failed to develop glycosuria for long periods while receiving diet 1 supplemented with inositol alone or with inositol and thiamine. In another experiment removal of inositol from an incomplete diet was followed by glycosuria, but this could only be controlled by completing the diet altogether. Similar evidence that inositol may have some preventive action is seen in period 10 of Table 1, when its withdrawal from a complete diet was promptly followed by glycosuria referable to some other cause. Besides being lipotropic, inositol can diminish the rate of digestion and absorption, but this effect has only been demonstrated with oral doses of 500 mg. per day (Bly, Heg-

geness, and Nasset, 1943). The dose which we gave was 40 mg. per day.

Findings which seem difficult to harmonize are:—1) striking evidence that glycosuria due to mild deficiency of water soluble vitamins occurs and that it responds to vitamin therapy; 2) difficulties in reproducing results at will in the same animal; 3) wide variation in the responses of different animals; and 4) suggestive evidence that the same vitamins slightly increase glycosuria during insulin deficiency. One must conclude from these results, and from the literature, that some effects of vitamins increase glycosuria while others diminish it, so that the net effect may vary greatly with the insulin supply and other circumstances. Since data for the dog are limited, information from experiments on rats is drawn upon in the following discussion.

Pantothenic acid deficiency in dogs greatly diminishes the rates of absorption of carbohydrate and protein, without notably altering total digestion and absorption (Bly, Heggeness, and Nasset, 1943). This would be a desirable change during insulin deficiency, in that absorption of actual and potential carbohydrate would be spread over a longer period. Thiamine and pyridoxin favorably influence absorption in rats (Leonards and Free, 1943) and the suggestion has been made that pyridoxin is involved in conversion of protein to carbohydrate (McHenry and Gavin, 1941). These effects would tend to increase glycosuria. But if the insulin supply is such that glucose absorbed or formed at normal rates is stored as glycogen until needed, then thiamine, riboflavin, pyridoxin, and pantothenate might all diminish glycosuria, for, apart from any effects upon oxidation, these factors promote conversion of carbohydrate to fat (Gavin and McHenry, 1941). Our most striking demonstrations of a relationship between vitamin intake and glycosuria were obtained in dog 33, which readily put on much larger amounts of subcutaneous fat than the other two dogs.

Those effects of vitamin deficiency which increase glycosuria could also be offset by any adverse effects that vitamin deficiency may have on functions of the pituitary or the adrenal cortex. Gaunt, Liling, and Mushett (1946) presented evidence that hypofunction of the adrenal cortex results from deficiency of pantothenic acid in rats.

SUMMARY

Losses of weight and nitrogen, and marked glycosuria, occurred in some depancreatized dogs during deficiency of water soluble vitamins, even though the food intake and the insulin dose remained constant, and choline or other lipotropic factors were supplied. Control of diabetes was reestablished when thiamine, riboflavin, nicotinic acid, pyridoxin, and pantothenic acid were added to the diet. Pyridoxin and pantothenic acid, separately and together, diminished glycosuria and improved the nitrogen balance in experiments in which

they were the only variables. One insulin-treated depancreatized animal never developed glycosuria when water soluble vitamins were withheld, and this situation was not altered by feeding succinylsulfathiazole. During insulin deficiency, glycosuria could not be diminished by completing the vitamin supplement, in fact glucose output usually increased slightly when essential factors were added. Possible reasons for this, and for the variability of results in different animals are discussed.

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EFFECT OF MAMMALIAN PITUITARY GONADOTROPINS ON OVULATION IN THE FROG, RANA PIPIENS[1]

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THE FIRST experiments on the induction of ovulation in Amphibia by implanting pituitary tissue were done by Wolf (1929) on *Rana pipiens* and Houssay, Giusti, and Lascano-Gonzalez (1929) on *Bufo marinus*. These observations have been confirmed and extended by many investigators since that time to include various other species of both Anura and Urodela (see review by Creaser and Gorbman, 1939). In fact, in many laboratories experimental ovulation of *Rana pipiens* has become a routine procedure for obtaining embryological material.

In general it has been found that the best results are obtained when the pituitary tissue implanted, or injected as extracts, belongs to the same species as the recipient. Ovulation in *R. pipiens* has been brought about by implanting pituitaries of other Amphibia, but the amount of pituitary tissue required is greater and the number of eggs ovulated is usually smaller than when homoplastic implants are used. The pituitaries of more distantly related vertebrates have been reported as being even less effective or without effect. So far as we are aware no one has found it possible to produce ovulation in *R. pipiens* by administering mammalian gonadotropins although a few instances of positive results have been reported for certain other Amphibia (Creaser and Gorbman, 1939). Therefore we should like to present the results of a series of experiments in which ovulation was induced both in intact frogs and fragments of ovaries *in vitro* by extracts of mammalian pituitary glands.

MATERIALS AND METHODS

Sheep pituitary glands were used as a source for gonadotropic preparations and included (1) a fraction containing the follicle-stimulating hormone (FSH) which in doses of 50 units did not show the presence of luteinizing hormone (LH). (2) a preparation containing FSH and LH in a proportion of one unit of FSH to two units of LH, (3) a combination of FSH and LH in a ratio of one unit of FSH to three units of LH, (4) an extract containing FSH and LH in a proportion of two units of FSH to one unit of LH, and (5) a crude alkaline extract of whole pituitary glands.[2]

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These preparations were standardized by the method described by Fevold (1939) and the ratios between FSH and LH used in the experiments were obtained by making dilutions and combinations of the different extracts. It should be mentioned that the unitages stated for unfractionated extracts must be considered as approximations, for exact quantitative determinations, especially in regard to FSH, cannot be made on mixtures due to the augmentation reaction between the two hormones.

The frogs used were large females weighing 50 to 100 grams and were obtained directly from hibernation. After arrival in the laboratory, the animals were kept under conditions simulating hibernation, *i.e.*, in a cold room (4°C.) in large aquaria containing wet sphagnum and covered by a dark cloth. The technique employed in the *in vitro* experiments has been described in detail in a recent paper (Wright, 1945). In brief, the ovaries were cut into small portions containing 15 to 30 eggs, and each piece was suspended by cotton thread in a stoppered vial with 10 cc. of Holtfreter's solution to which was added the desired amount of pituitary substance as a triturated suspension or extract. Frogs under light ether anesthesia were hypophysectomized through the roof of the mouth by drilling a hole in the basisphenoid bone with a 2-mm. trephine, and sucking out the anterior lobe into a pipette.

In estimating percentages of ovulation *in vivo*, the number of eggs ovulated (*i.e.*, in the body cavity, oviducts, or uteri) was compared with the number of eggs still remaining in the ovary, and thus the total present in the ovaries before ovulation started was readily determined. Percentages of ovulation *in vitro* were determined by dividing the number of eggs ovulated (*i.e.*, fallen to the bottoms of the vials) by the total number of eggs in the ovarian fragments before ovulation began. All experiments, both *in vivo* and *in vitro*, were conducted at room temperature (22°–24°C.).

EXPERIMENTAL PROCEDURES AND RESULTS

It was found that FSH was capable of inducing ovulation in the normal adult frog during January, February, and March, the three

TABLE I. RESPONSE OF NORMAL FROGS IN MARCH TO INTRAPERITONEAL INJECTIONS OF FSH. PERCENTAGES OF OVULATION IN ALL FROGS WERE DETERMINED BY LAPAROTOMY 32 HOURS AFTER THE LAST INJECTION, AND THE AVERAGES SHOWN WERE COMPUTED ON THE BASIS OF ONLY THOSE FROGS WHICH OVULATED

Daily dosage—units FSH	No. days injected	No. frogs injected	No. positive response	Average percent ovulation
2 units	2	4	1	0.75%
3 units	2	4	3	4.0%
3½ units	2	2	1	4.0%
5 units	2	4	3	50.0%
7½ units	2	4	4	95.0%
9 units	2	4	4	99.5%

months in which these experiments were performed. A series of 22 normal females were given various amounts of FSH in two equal daily doses, and 32 hours after the last injection, the number of eggs

ovulated in each frog was determined by laparotomy. The results obtained (Table I) show a good correlation between dosage and response. In a few additional experiments the length of the treatment was varied, the results of which indicate that the percentage of eggs ovulated may be influenced by both the dosage and the length of time it is administered. Four females ovulated 100% of their eggs after receiving three daily injections of 5 units FSH, while two frogs given 3 units FSH daily for five days ovulated only 10%. One female given 2 units a day for six days ovulated but twelve eggs while another given 1 unit FSH daily for the same length of time did not ovulate.

From these results, it cannot be stated definitely whether the FSH acted directly on the ovary causing ovulation, or whether the preparation was effective by way of, or with the aid of, the recipient's pituitary gland. It seemed important, therefore, to test the preparation on hypophysectomized frogs. Two hypophysectomized females were given daily injections of 5 units FSH, beginning immediately after removal of the anterior lobe, and continuing for six days. No ovulation had occurred when laparotomies were made two days later. However, when these two animals, which had received a total of 30 units each of FSH, were given the equivalent of one frog pituitary gland on the eighth day after hypophysectomy, they ovulated 100% of their eggs. Control, hypophysectomized frogs kept at room temperature for eight days did not ovulate in response to the injection of one pituitary gland. Similarly four other hypophysectomized frogs given daily injections of 5 units FSH for three days did not respond, but when a frog pituitary gland was administered on the fifth day, complete ovulation was obtained. After control, hypophysectomized females had remained at room temperature for four days, they ovulated only a few (5 to 10%) of their eggs in response to the equivalent of one frog pituitary gland.

These data indicate that FSH does not produce ovulation in hypophysectomized frogs but does have the ability to maintain the ovary in a highly sensitized condition so that it will respond readily to the injection of frog pituitary glands. Further evidence for the sensitizing action of FSH is shown by an experiment such as the following: The left ovary of a frog weighing 64.0 grams was removed after lightly anesthetizing the animal with ether. Pieces of this ovary did not ovulate within 32 hours when treated *in vitro* with $\frac{1}{8}$ of a female anterior lobe. Immediately after removal of the left ovary, the frog was given $2\frac{1}{2}$ units of FSH intraperitoneally. Sixteen hours later the right ovary was removed and ovarian fragments were placed in suspensions of $\frac{1}{8}$ pituitary identical to those used the previous day on the left ovary. Ovulation at the end of 32 hours was 14.4%. Portions of the sensitized ovary in Holtfreter's fluid did not ovulate. It might seem that this experiment would have been more conclusive had an hypophysectomized frog been used. In actuality such animals appear

to be impractical for an experiment of this sort, because the process of hypophysectomy itself brings about a temporary sensitization of the ovary (Wright, 1946). Therefore, it would be difficult to ascertain how much, if any, of the sensitization observed was due to the FSH injected.

It was of considerable additional interest to find that FSH could

TABLE II. RESPONSE OF HYPOPHYSECTOMIZED FROGS TO INTRAPERITONEAL INJECTIONS OF FSH AND LH FROM SHEEP PITUITARY GLANDS. IN ALL ANIMALS INJECTIONS WERE BEGUN 16 HOURS AFTER HYPOPHYSECTOMY, AND PERCENTAGES OF OVULATION WERE DETERMINED BY LAPAROTOMY 32 HOURS AFTER THE LAST INJECTION. EXPERIMENTS CONDUCTED FEBRUARY 1 TO MARCH 11

Daily dosage units LH units FSH	No. days injected	No. frogs injected	No. positive response	Average percent ovulation
1 u. LH ½ u. FSH	2	4	4	2.0%
2 u. LH 1 u. FSH	2	5	5	5.0%
3 u. LH 1½ u. FSH	2	4	4	2.0%
5 u. LH 2½ u. FSH	1	4	4	25.0%
7½ u. LH 3¾ u. FSH	1	4	3	22.0%
8 u. LH 4 u. FSH	1	1	1	10.0%
8½ u. LH 4¼ u. FSH	1	2	2	90.0%
9 u. LH 4½ u. FSH	1	4	4	5.0%
10 u. LH 5 u. FSH	1	4	4	95.0%
8 u. LH 4 u. FSH	2	1	1	98.0%
10 u. LH 5 u. FSH	2	2	2	99.5%

sensitize ovarian tissue *in vitro*. Bits of the ovary of a normal frog weighing 67 grams placed in suspensions of $\frac{1}{8}$ anterior lobe did not ovulate in 32 hours. Other fragments of this ovary were placed concurrently in a series of vials containing $\frac{1}{4}$ unit of FSH. Eighteen hours later these ovarian bits were transferred from the FSH solution to suspensions of $\frac{1}{8}$ anterior pituitary, identical to those used the previous day on bits of the same ovary. Ovulation after 32 hours in the

pituitary suspensions was 43.3%. Control fragments which remained in FSH solution did not ovulate.

It seems probable that in the frog the chief function of FSH in the process of ovulation is one of sensitizing the follicle. Although ovulation in normal frogs can be induced by the injection of FSH, hypophysectomized animals do not respond. In contrast with this, a combination of FSH and LH in appropriate dosage will induce ovulation in both hypophysectomized and normal frogs (Tables II and III). This suggests the possibility that ovulation in the normal frog in response to FSH is made possible by the contribution of LH from the

TABLE III. RESPONSE OF NORMAL FROGS TO INTRAPERITONEAL INJECTIONS OF FSH AND LH FROM SHEEP PITUITARY GLANDS. PERCENTAGES OF OVULATION IN ALL FROGS WERE DETERMINED BY LAPAROTOMY 32 HOURS AFTER THE LAST INJECTION, AND THE AVERAGES SHOWN WERE COMPUTED ON THE BASIS OF ONLY THOSE ANIMALS WHICH OVULATED. EXPERIMENTS CONDUCTED JANUARY 27 TO MARCH 7

Daily dosage units LH units FSH	No. days injected	No. frogs injected	No. positive response	Average percent ovulation
1 u. LH ½ u. FSH	2	4	2	1.5%
2 u. LH 1 u. FSH	2	5	2	3.0%
3½ u. LH 1¾ u. FSH	2	4	3	33.0%
5 u. LH 2½ u. FSH	2	4	4	54.0%
7½ u. LH 3¾ u. FSH	2	4	4	93.0%
10 u. LH 5 u. FSH	2	3	2	95.0%
10 u. LH 5 u. FSH	3	4	4	99.5%

animal's own pituitary gland. With this idea in mind, experiments were conducted to test the relationship between FSH and LH in the ovulatory mechanism. The data in Tables II and III show that hypophysectomized animals are decidedly more sensitive than normal to equivalent dosages of FSH and LH, but are much more variable in their responses; in fact, there is no consistent correlation between dosage and response except in a very general way. Although the normal animal is less sensitive than the hypophysectomized frog, its response is by far more predictable. It is known that hypophysectomized frogs are more sensitive than normal animals to the injection of equivalent doses of frog pituitary glands (Rugh, 1939), and, therefore, it is not surprising that they respond in a similar way to mam-

malian pituitary substances. It has been suggested (Wright, 1946) that the explanation for the increased sensitivity of hypophysectomized frogs lies in the release of an hypophyseal sensitizing factor (presumably FSH) during the process of removal of the anterior lobe. Since no two operations of this sort can be conducted identically, it will naturally follow that the release of hormone from the pituitary (and consequent sensitization of the ovary) will vary from one animal

TABLE IV. RESPONSE OF PORTIONS OF NORMAL OVARIES TO MAMMALIAN FSH AND LH *in vitro*. ALL EXPERIMENTS RECORDED AS OF A GIVEN DATE WERE CONDUCTED ON MATERIAL FROM THE SAME OVARY. PERCENATGES OF OVULATION DETERMINED 32 HOURS AFTER THE BEGINNING OF THE EXPERIMENT

Date	units LH units FSH in 10 cc.	Percentage ovulation FSH and LH	Percentage ovulation $\frac{1}{8}$ frog pituitary	Percentage ovulation $\frac{1}{16}$ frog pituitary
2/17	1 u. LH $\frac{1}{2}$ u. FSH	7.6%	46.8%	
	$\frac{1}{2}$ u. LH $\frac{1}{4}$ u. FSH	1.1%		
2/29	1 u. LH $\frac{1}{2}$ u. FSH	64.1%		48.0%
3/2	1 u. LH $\frac{1}{2}$ u. FSH	46.2%	56.8%	
	$\frac{1}{2}$ u. LH $\frac{1}{4}$ u. FSH	48.9%		
	$\frac{1}{4}$ u. LH $\frac{1}{8}$ u. FSH	6.1%		
3/7	1 u. LH $\frac{1}{2}$ u. FSH	42.5%		64.0%
	$\frac{1}{2}$ u. LH $\frac{1}{4}$ u. FSH	38.7%		

to another. Very likely, then, the inconsistency in response of hypophysectomized frogs to FSH and LH may be due directly to the release of different amounts of the sensitizing agent resulting from unavoidable variations in the technique of anterior lobe removal.

The fact that a combination of FSH and LH causes ovulation in hypophysectomized frogs is convincing proof that these hormones act without mediation of the animal's pituitary gland. That these substances act directly on the ovarian tissue is made even more striking by the observation that FSH and LH will cause ovulation *in vitro* (Table IV) and that FSH, as in the intact animal, greatly facilitates the reaction. Ovulation from portions of a normal ovary *in vitro* has not been obtained with more dilute solutions than $\frac{1}{4}$ unit LH plus $\frac{1}{8}$ unit FSH, but fragments of ovaries sensitized *in vitro* with FSH have responded (37.4%) to as little as 1/16 unit LH plus 1/32 unit FSH.

It seems probable that a combination of the two pituitary hormones, FSH and LH, are required for ovulation in the frog. It is also of interest that the results of these experiments on the frog are in remarkable agreement with those of similar studies on mammals (Foster, Foster, and Hisaw, 1937). Hypophysectomized rabbits in which follicular development has been induced by subcutaneous injections of FSH did not ovulate when given intravenous injections of FSH or LH but did respond to a mixture of the two hormones. Unfortunately, an LH preparation of sufficient purity to determine the independent action of this hormone on ovulation in the frog was not available. However, it is clear that FSH alone will not induce ovulation in hypophysectomized frogs or in ovarian fragments *in vitro* while preparations containing both FSH and LH are effective. The optimal balance between the two hormones that is most favorable for ovulation was not determined but our results indicate that an adequate amount of FSH must be present along with LH in order for ovulation to take place. For example, ovulation did not occur in vials which contained $\frac{1}{2}$ unit LH and $\frac{1}{8}$ unit FSH but did take place (16.7%) when $\frac{1}{2}$ unit LH was combined with 1 unit FSH.

It is difficult to find a satisfactory explanation for the failure of other investigators to produce ovulation in *Rana pipiens* with mammalian pituitary extracts. While it is probably true that there is an optimal balance between FSH and LH which is most favorable for ovulation, yet we found it possible to induce ovulation in normal frogs by giving an unfractionated extract prepared by our method. This preparation of sheep pituitary glands contained approximately one unit each of FSH and LH per cc. The experiments were performed in November on six frogs each of which was given three injections of the pituitary extract twelve hours apart and totaling 5.5 to 7.0 cc. Ovulation occurred in all of the six animals within 48 hours after the last dose was given and the average percentage of ovulation was 22.5%.

DISCUSSION

There seems quite general agreement that follicular development and ovulation in mammals is controlled and regulated by the pituitary gonadotropic hormones, FSH and LH. It also seems probable that this is equally true of all vertebrate animals. These gonadotropic hormones as such have not been isolated from the pituitaries of frogs or other cold-blooded vertebrates but the efforts of hypophysectomy and replacement therapy by means of implants and unfractionated extracts strongly suggest their presence. Partially fractionated preparations of mammalian FSH and LH have been tested on several species of amphibia and in certain instances ovulation has been induced (Creaser and Gorbman, 1939). However, in *Rana pipiens* such experiments have been unsuccessful. The only exception we have found is a reference by Adams and Granger (1938) to unpublished

work by H. O'Neil who obtained ovulation in *Rana pipiens* with an extract of beef pituitaries (Phyone).

The data presented in this paper show that the gonadotropic hormones of the mammalian pituitary are as effective in promoting ovulation as material from frog pituitary glands. Furthermore, the mammalian hormones seem to act on the frog's ovary as they do in mammals, *i.e.*, FSH cannot produce ovulation in either hypophysectomized rabbits or frogs, and apparently LH must be present. The data at hand are not sufficient to establish the exact quantitative relationship between FSH and LH required to produce maximal ovulation in the frog, but they are adequate to suggest that a quantitative relationship exists.

It is not certain what significance should be attached to the sensitization of the ovary by an FSH preparation. It may imply that FSH not only promotes follicular growth but also conditions the follicle in a way that facilitates ovulation. It also may have some relationship to the progressive increase in responsiveness of the frog's ovaries during the months between early fall and spring. If so, this would indicate that FSH is being secreted by the animal's pituitary during hibernation.

It is not known what changes occur in the pituitary gland at the time of emergence from hibernation to bring about the most suitable balance between FSH and LH for ovulation. This might be accomplished by an increase in output of LH by the pituitary gland. Also, it seems possible that the increased sensitivity due to FSH action could be sufficient to enable the follicle to respond to an existing concentration of LH. This may be the reason why injections of FSH cause ovulation in the normal animal and are ineffective after hypophysectomy.

SUMMARY

Fractionated extracts of sheep pituitary glands were found effective for inducing ovulation in adult frogs (*Rana pipiens*) and in bits of the ovary *in vitro*. A preparation of FSH produced ovulation in normal frogs but did not cause ovulation in the hypophysectomized animal or in ovarian fragments *in vitro*. However, in both the hypophysectomized frog and in the *in vitro* experiments, the FSH preparation brought about a sensitization of the follicles so that they responded more readily than normal to an appropriate ovulating stimulus.

A combination of FSH and LH caused ovulation in normal and hypophysectomized frogs and in ovarian fragments *in vitro*. Hypophysectomized frogs were the more sensitive to equivalent doses of FSH and LH, but the response of normal frogs was more predictable. A crude alkaline extract of sheep pituitary glands also induced ovulation in normal frogs.

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QUANTITATIVE STUDIES OF THE OPENING OF THE VAGINA OF IMMATURE RATS FOLLOWING INJECTION OF FEMALE BLOOD AND URINE

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RECENTLY Hartman and Littrell (1945) reported a new test for estrogens in minute amounts of blood. This test was based on the ability of estrogens to open the vagina of 16 to 21 day old rats. Their method was said to be sufficiently sensitive to detect the amount of estrogen in 0.02 cc. of blood from the finger of normally menstruating women. The reported simplicity and sensitivity of this test made it seem advisable to standardize it for use in estrogen assays. However, when studies similar to those of Hartman and Littrell (1945) were conducted in this laboratory, it became evident that the estrogen content of blood and urine was insufficient to be detected by this method. Therefore, an attempt was made to enhance its sensitivity by priming the animal with small doses of estrogens injected subcutaneously.

METHODS

Three hundred and eighty-seven immature female rats of the Sprague-Dawley strain were used. Forty-eight of these remained uninjected and served as controls. Litter mates were always used for the same dose level. A diet of standard laboratory chow was fed.

Experimental animals were injected subcutaneously in the region of the future vaginal opening with 0.02 cc. of the test substance. The doses used were chosen because they fell in or near the range to which castrate rats are generally sensitive in the usual method of estrogen assay. Whole blood and serum of women in various phases of the menstrual cycle, and of a patient with a proven granulosa cell tumor, were injected paravaginally. A few animals were also injected with urine of a patient with a granulosa cell tumor, of a hypogonadal female receiving estrogen therapy, of a patient with Cushing's disease and of a hypogonadal male receiving androgen therapy. Estrone and estriol, in sesame oil, were used as standard estrogens.

The rats to be primed received, subcutaneously, an amount of estrogen which was slightly smaller than the minimal effective subcutaneous dose which will open the vagina of an immature rat. In addition, two groups of rats received, paravaginally, multiple injections of standard estrone solutions. Estrone was used for the standard in the majority of experiments, but

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estriol was used in four groups because of its reported specific potency in opening the rat's vagina (Dingemanse, 1937). The animals were examined daily until the 32nd day of life, but not thereafter as it seemed that opening of the vagina after this interval was not significant in the light of experience with control animals.

An animal was designated as positive as soon as a pin-point hole appeared in the region of the vaginal opening. Generally, it was possible to squeeze a small amount of fluid from the opening by gentle pressure.

RESULTS

In forty-eight control animals, opening of the vagina had occurred by the 32nd day in only one rat (table 1). The vaginas of eighteen rats opened between the 36th and 40th day; a total of 23 had opened by the 45th day.

Assays of Standard Solutions: It will be noted from Table 1 that the smallest effective amounts of estrogens were .00025 mg. estrone

TABLE I. PARAVAGINAL INJECTION OF ESTRONE AND ESTRIOL

Substance	Day of life injected	No. of rats	Day after injection 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Estrone .0025 mg.	19	5	—[1]	—	—	—	—	—	3+[2]	3+	3+	3+	3+	3+	3+	3+	3+	3+
Estrone .0012 mg.	24	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estrone .0005 mg.	24	4	—	—	—	—	2+	2+	2+	2+	4+							
Estrone .0005 mg.	17	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estrone .00025 mg.	17	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estrone .00025 mg.	21	4	—	—	—	—	—	—	—	—	—	2+	3+	3+	3+	3+	3+	3+
Estrone .00025 mg.	20	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estriol .0004 mg.	17	5	—	—	—	—	2+	2+	3+	3+	3+	5+						
Estriol .0004 mg.	18	5	—	—	—	—	—	—	—	—	1+	1+	1+	1+	1+	1+	2+	2+
Estriol .0002 mg.	17	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estriol .0002 mg.	18	6	—	—	—	—	—	—	—	—	1+	1+	1+	1+	1+	4+	4+	4+
Estriol .0002 mg.	16	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Estriol .0001 mg.	16	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[1] Dash signifies all rats were negative.
[2] Number of rats positive in a group.

and .0002 mg. estriol. However, it is also evident from the table that neither of these results was reproducible and that even higher dosages at times did not give positive results.

One group of eight rats was injected with .00006 mg. of estrone every day for four days and another group of seven was injected with .00006 mg. estrone every other day for a total of three injections. There was no premature opening of the vagina of the rats in either group.

A total of 102 rats was used to determine the priming dose, *i.e.*,

the largest dose which could be given subcutaneously without opening the immature rat's vagina. This dose was found to be .00025 mg. of estrone.

By comparing Tables 1 and 2, it is obvious that priming increased the sensitivity. However, it is also apparent that the response was not constant.

Assays of Whole Blood, Serum and Urine: Whole blood and serum of ten normal young women, who were in various phases of the

TABLE II. SUBCUTANEOUS PRIMING FOLLOWED BY PARAVAGINAL INJECTION
(Rats were primed with .00025 mg. estrone subcutaneously)

Paravaginal dose	Day of life of priming	Day of life of paravaginal injection	No. of rats	Day after injection												
				1	2	3	4	5	6	7	8	9	10	11	12	13
Estrone .0001 mg.	20	21	5	2+[2]	2+	2+	2+	4+	4+	4+	5+					
Estrone .0001 mg.	20	21	8	—[1]	—	—	—	—	—	—	—	—	—	—	—	—
Estrone .00005 mg.	20	21	5	—	—	—	—	—	—	—	—	1+	1+	1+	1+	1+
Estrone .00005 mg.	18	19	4	—	—	—	—	—	—	—	—	—	—	—	—	—
Estrone .00005 mg.	18	19	4	—	1+	1+	2+	2+	2+	2+	2+	3+	3+	3+	3+	3+
Estrone .000025 mg.	18	19	6	—	—	—	—	—	—	—	—	—	—	—	—	—

[1] Dash signifies all rats were negative.
[2] Number of rats positive in a group.

menstrual cycle, when injected paravaginally into rats in doses of .02 cc., did not cause opening of the vagina. Whole blood from a normally menstruating female, injected subcutaneously in single .75 cc. doses to four rats, yielded no response.

Whole blood, serum, and hydrolyzed and unhydrolyzed urine of a patient with a proven granulosa cell tumor, who two months previously had a urinary excretion of 166 castrate mouse units per 24 hours, failed to cause opening of the vagina.

Urine in .02 cc. amounts from a male patient with Cushing's disease, a hypogonadal female receiving estrogen therapy (.05 mg. Estinyl/day), and a hypogonadal male receiving 25 mg. of testosterone propionate every 2 days also gave negative results.

Large (.5 to 2 cc.) subcutaneous injections of hydrolyzed and unhydrolyzed urine from the patient with granulosa cell tumor and from the hypogonadal male gave no vaginal response.

DISCUSSION

The opening of the vagina of immature rats following the paravaginal injection of estrogens was found to be little, if any, more sensitive as an assay method than is the test using the response in the vaginal smear of the castrate rat or mouse. There was opening of the vagina in some of the rats injected paravaginally with .0005 mg. of estrone, but this dosage failed to open the vagina in any of the rats of

another group. There was opening in only 3 of 15 rats injected with .00025 mg. of estrone paravaginally. These dose levels are of the magnitude generally considered to be about one castrate rat or mouse unit. The sensitivity to estriol was only slightly greater than is present with the usual castrate rat or mouse.

The premature opening of the vagina following estrogen stimulation was not a quantitative phenomenon. There was great individual variation among animals, both of the same and different litters. When very dilute solutions of estrogen were used, on some occasions part of the group responded with fairly rapid opening, while others did not open until the control group did. Testing at the same dose level, repeated on several occasions, often gave negative responses after a previous positive result.

Previous priming with subcutaneous estrogen increased the sensitivity of the paravaginal route, but this technique also yielded a greatly varied response.

Comparisons of standard solutions of estrogens indicate that the rats used in this laboratory were at least as sensitive in their vaginal response as were those of Hartman and Littrell (1945). However, we were unable to confirm the results of Hartman and Littrell (1945) using .02 cc. of midcycle female blood.

Moreover, on the basis of previous studies it would seem that the quantity of estrogens in 0.02 cc. of blood, the amount recommended for injection, is greatly inadequate to produce vaginal opening. For example, .00025 mg. or more of estrone was necessary to promote vaginal opening, yet Hartman and Littrell (1945) calculate, on the basis of Markee and Berg's (1944) observations, that only .0000001 mg. of estrogens is found in .02 cc. of normal female blood. Furthermore, on the basis of reports of other authors (Frank and Goldberger, 1945, Janney, 1929, Ford and Mueller, 1932), one can expect to find about 1 mouse unit per 40 cc. of blood at some time during the menstrual cycle. Since a positive test in our animals requires about 1 mouse unit of estrogen, it is obvious that the amount of estrogen in .02 cc. of blood is much too small to give a reaction.

A similar situation is found in relation to the estrogen content of urine. Kurzrok and Ratner (1932) found 30 rat units/liter, Werner (1941) 5 to 50 rat units/24 hrs. and D'Amour (1943) 1000 international units/24 hrs. of estrogen in the urine of normally menstruating women, the highest levels being found in midcycle. However, using these determinations, the estrogen content of .02 cc. of urine is much too low to be detected by the method of Hartman and Littrell.

SUMMARY

In an attempt to use the technique of Hartman and Littrell (1945) for estrogen assay, rats from 16 to 24 days of age were injected para-

vaginally with various concentrations of estrone, estriol, human blood, serum and urine, and the time of opening of the vagina was noted.

Rats were also injected subcutaneously with the same substances.

It was found that .00025 mg. or more of estrone, given paravaginally, was required to open prematurely the vagina of rats, and that subcutaneous injection was less sensitive.

Priming the rats with a small subcutaneous injection of estrone, followed by paravaginal injection, increased slightly the sensitivity to paravaginal injection. However, the inconstancy of results and lack of sensitivity of this method preclude its use as a quantitative assay method.

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A TURBIDIMETRIC METHOD FOR DETERMINING HYALURONIDASE IN SEMEN AND TISSUE EXTRACTS[1]

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RECENT EVIDENCE (McClean and Rowlands, 1942; Fekete and Duran-Reynals, 1943; Rowlands, 1944; Joel and Eichenberger, 1945; Werthessen et al., 1945; Leonard and Kurzrok, 1945) of the possible importance of hyaluronidase in the process of fertilization made it desirable that a simple, rapid method adaptable to routine determination of the enzyme be available. A quantitative biological test was recently proposed by Leonard and Kurzrok (1946). This was not found satisfactory when large numbers of assays were required to be made in one day.

To date the available non-biological techniques for hyaluronidase determination consist of (1) measurement of the reducing substances formed by the hydrolysis of the glucosidic linkages of hyaluronic acid (Meyer, 1946), (2) the measurement of the decrease in viscosity due to enzymatic action on a given hyaluronic acid solution (Madinaveitia and Quibell, 1940), or (3) the measurement of the turbidity produced by the interaction of acidified blood serum with the hyaluronic acid polymer (Seastone 1939, 1943; and Kass and Seastone, 1944). Because the first two procedures appeared to involve laborious and time-consuming manipulations, their application on a large scale seemed restricted. This consideration suggested an investigation of the turbidimetric technique.

The turbidimetric method as developed by Seastone (1939, 1943) and Kass and Seastone (1944) is based on the observation of Meyer and Palmer (1936) that the turbidity produced by the interaction of hyaluronic acid and acidified blood serum is, within limits, a function of the hyaluronic acid concentration.

The procedure as described below is a modification of the method of Kass and Seastone. Considerable flexibility has been effected by revising the manipulative technique, through further application of photoelectric turbidimetry, and by further investigation of the experimental conditions under which the determination may best be

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[2] Fellow of the Schering Corporation.

carried out. The resulting turbidity is measured in a photoelectric colorimeter and the scale-units of the colorimeter may then be translated into either hyaluronidase units by measuring the degree of turbidity reduction as compared with a standard, or as milligrams of hyaluronic acid depolymerized. The adaptation of this method to certain sources of the enzyme, namely, semen and testicular tissue is presented in this study.

METHOD

Reagents

Hyaluronic acid solution. Dissolve 20.0 mg. hyaluronic acid in 50 ml. 0.1M acetate buffer (*p*H 6.0) and warm the solution for several hours at approximately 37° C. to effect complete solution. Centrifuge or filter the solution to remove lint or other particles. The solution is stable for several weeks if stored at low temperature (10° C).

Hyaluronic acid may be obtained from several sources: from vitreous humor of cattle eyes, synovial fluid, or human umbilical cords (Meyer and Palmer, 1936; McClean, 1943). Vitreous humor was most satisfactory in this work because of availability and ease of preparation.

0.5M acetic acid.

0.5M sodium acetate solution.

0.1M acetate buffer (pH 6.0). Mix one part 0.5M acetic acid with 20 parts 0.5M sodium acetate solution and dilute the solution 1:5.

0.5M acetate buffer (pH 4.2). Mix 28 parts 0.5M acetic acid with 10 parts 0.5M sodium acetate solution.

Acidified blood serum. Add 5 ml. of fresh horse, rabbit, or sheep serum to 45 ml. 0.5M acetate buffer (*p*H 4.2) and adjust the *p*H of the solution to 4.2 with the glass electrode. The solution should stand for at least 24 hours before use.

It is best that only fresh serum (no more than 2–3 weeks old) be used because the serum, acidified or unacidified, undergoes a change which produces lowered, and therefore frequently unusable turbidity values.

Apparatus

Colorimeter. For determining turbidity, a Klett-Summerson Photoelectric Colorimeter with red filter No. 66 was used.

Colorimeter tubes. It is imperative that carefully matched tubes be employed. The so-called "matched" tubes obtainable from supply houses may vary as much as 10 scale units. To circumvent this situation, one tube was selected as the reference tube and all others were compared to it. The tubes were classified into the following 3 groups: those exhibiting differences of 0–3, 4–6, and 7–8 scale-units. Determinations usually were made in tubes of one group, otherwise appropriate correction factors were introduced.

Oven. The enzymatic reactions were carried out in an oven at a temperature of 37° C.

Turbidimetric technique

The following procedure is general and is the one upon which the specific determinations described in this paper are based. Specific applications are presented in illustrative experiments.

Meyer (1936) and Kass and Seastone (1944) have shown that under certain well-defined conditions, the turbidity produced when hyaluronic acid is mixed with acidified blood serum is a function of the hyaluronic acid concentration. In accordance with Kass and Seastone, the hyaluronidase unit is designated the *turbidity-reducing unit* (TRU) and is defined as *the amount of enzyme which will reduce the turbidity produced by 0.2 mg. hyaluronic acid to the equivalent turbidity of 0.1 mg. hyaluronic acid in 30 minutes and at 37° C.*

To assay a given enzyme preparation, the enzyme solution of selected concentration and the hyaluronic acid solution were brought to 37° C. In a colorimeter tube 0.5 ml. of the enzyme solution and 0.5 ml. of substrate (containing 0.2 mg. of hyaluronic acid) were mixed well and incubated at 37° C. for 3.0 minutes. The tubes were then chilled in an ice-bath for five minutes and 30 ml. *p*H 4.2 acetate buffer followed by 1 ml. acidified blood serum were added. The contents of the tube were mixed by inverting the tube three times. The turbidity was permitted to develop at room temperature for 30 minutes and was then measured in the colorimeter. The *turbidity value* is the number of scale-divisions read on the colorimeter scale.

In order to equate the scale reading obtained to a known turbidity value, it is required that two standards be prepared. These are designated as the *0.2 mg. standard* and the *0.1 mg. standard.* They are prepared during the running of the determination and for any one day's work it is only necessary that the values of these standards be determined once. The 0.2 mg. standard was prepared by placing 0.5 ml. substrate solution in a colorimeter tube, and adding, in order: 0.5 ml. *p*H 6.0 acetate buffer, 3.0 ml. *p*H 4.2 acetate buffer, and 1 ml. acidified blood serum. The 0.1 mg. standard was prepared in a similar manner excepting that 0.25 ml. substrate solution and 0.75 ml. *p*H 6.0 acetate buffer were used instead. The turbidity value was read at the end of thirty minutes. The scale readings correspond to the turbidities of 0.2 and 0.1 mg. hyaluronic acid and that concentration of enzyme which reduces the turbidity produced by 0.2 mg. hyaluronic acid to the turbidity produced by 0.1 mg. hyaluronic acid is said to contain one turbidity-reducing unit.

EXPERIMENTAL

Assay of bull testis-hyaluronidase in turbidity-reducing units (TRU)

In order to determine the activity of a powdered preparation of bull testis hyaluronidase, the following procedure was adopted. The enzyme solution of selected concentration and the substrate solution were warmed to 37° C. One ml. of enzyme solution was pipetted into a 3 ml. test tube and 1 ml. substrate solution added. The contents were mixed and incubated for 30 minutes. At the end of this period the tube was chilled in an ice bath for 5 minutes and then 1 ml. of the reaction mixture pipetted into a colorimeter tube which contained 3 ml. acetate buffer (*p*H 4.2) and 1 ml. acidified blood serum. The turbidity value was then obtained as previously described.

Turbidity values (to the nearest whole scale unit) were obtained for a total of six enzyme concentrations, repeating each determination 5 times. The average turbidity value for each enzyme concentration was computed and plotted against the corresponding enzyme

concentration. The results showed that in the 5 determinations the average error of a single observation never exceeded ± 1 scale division.

Figure 1 is the curve derived from this study and is the curve from which the activity and number of TRU of the preparation was computed. That point at which the curve intersects the 0.1 mg. standard level determines the degree of activity of the preparation. In this case 1 TRU was equivalent to 0.033 mg. of the preparation. The 0.2

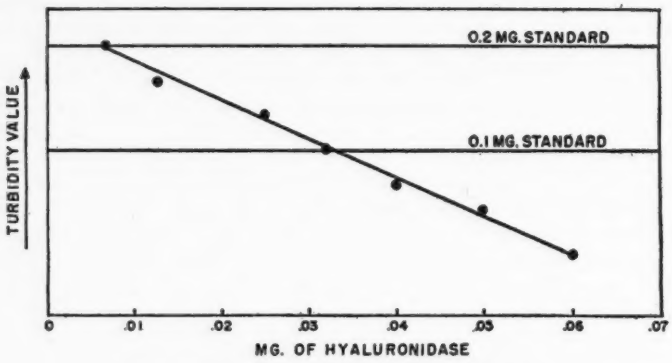

FIG. 1. Assay of Hyaluronidase Preparation A.

mg. standard in all experiments served as a check on the reacting substances in the test. Unless the instrument reading of at least 35 scale divisions was produced by the turbidity of the 0.2 mg. standard a check was made on the reacting substances.

Hyaluronidase activity in semen

Bull semen. The hyaluronidase concentration in seminal plasma, i.e., fluid with sperm removed, was determined within 6–48 hours following ejaculation. Preliminary experiments indicated that the enzyme concentration of a given semen sample exhibited no change over a period of 3–4 days provided the semen was stored under refrigeration (10° C.).

The method of determining hyaluronidase in the seminal plasma consisted of adding 0.25 ml. well mixed semen to 2.25 ml. acetate buffer (*p*H 6.0) in a 3 ml. test tube, mixing the contents by inverting the tube several times, and then centrifuging the mixture for 20 minutes at 2,000 rpm. Varying dilutions of the clear supernatant were then prepared in *p*H 6.0 acetate buffer and *0.5 ml.* aliquots of the dilutions reacted with 0.5 ml. substrate solution. Turbidity values were then determined in the usual manner. A blank was prepared for each semen specimen by placing 0.5 ml. of the lowest dilution tested in a colorimeter tube containing 0.5 ml. *p*H 6.0 buffer, 3 ml. *p*H 4.2 buffer, and 1 ml. acidified blood serum. Because the semen

was found to contribute no interfering substances, the blank is substantially a reagent blank. In the course of several hundred semen assays, the absolute turbidity value of any one blank never exceeded 4 scale units. Therefore, it seemed sufficient to prepare only one blank for any one series of semen dilutions.

To calculate the number of TRU per ml. of plasma, the turbidity values obtained were plotted against their respective dilutions. From that point at which the 0.1 mg. standard level intersects the curve the effective dilution was determined. Figure 2 shows the graphs of

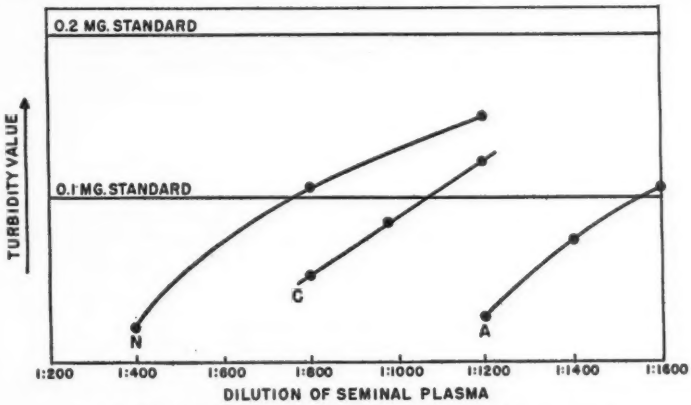

FIG. 2. Typical Curves of Bull Seman Hyaluronidase Assays.

three semen assays. To compute the number of TRU per ml. of semen, multiply the effective dilution by 2 since only 0.5 ml. aliquot was used in the determination. For example, the effective dilution of A (fig. 2) was 1:1,550; therefore this sample contained 3,100 TRU per ml.

Human semen. The technique for determining hyaluronidase in human semen is similar in principle to that described for bull semen. Human semen, however, contains a lower concentration of enzyme and the dilutions employed were of the magnitude of 1:10 to 1:100. Also, human seminal plasma is quite persistently turbid after the removal of the sperm and it was necessary to use correction blanks for each of the dilutions used.[3] In such samples, it has been customary to filter the centrifuged diluted semen before continuing with the determination in order to remove extraneous particles at the surface of the liquid. In Table 1 a protocol of a typical semen assay is given

[3] During the course of carrying out human semen hyaluronidase determinations it was found that if 0.1 ml. toluene was added to each ml. of semen in the sample, maintenance of enzyme activity was possible for at least 5 days at room temperature. Untreated samples exhibited marked decrease in activity under similar conditions. This observation made it possible for one of us (R.K.) to send samples through the mails from New York City to Ithaca, N. Y. for hyaluronidase assay.

together with the method of calculating the enzyme concentration.

Tissue. Among the problems concerned with hyaluronidase is the quantitative determination of the enzyme in small amounts of testicular tissue from animals under different experimental conditions or from other tissues. Extracts of rat and chicken testes and rat salivary glands were prepared by modifications of the method of Kass and Seastone (1944). A Potter homogenizer was used to mince the tissue and was made for us by Dr. H. Albaum of New York.

Approximately 5–10 grams of the tissue, weighed to the nearest 0.1 gm., was homogenized and the resulting mince centrifuged. The

TABLE 1. HYALURONIDASE ASSAY OF HUMAN SEMEN
0.25 ml. semen + 2.00 ml. *p*H 6.0 acetate buffer. Centrifuge. Filter. 1 ml. of filtrate diluted to 2 ml. Final dilution is 1:18.

Volume of 1:18 dilution used (ml.)						
	0.5		0.25		0.1	
*p*H 6.0 buffer (ml.)			0.25	0.25	0.40	0.40
Substrate solution (ml.)	0.50	0.50	0.50	0.50	0.50	0.50
Add following volume (ml.) to each tube; incubate 30 minutes and chill for 5 minutes.						
*p*H 4.2 buffer (ml.)	3.00	4.00	3.00	4.00	3.00	4.00
Acid blood serum	1.00		1.00		1.00	
Mix. Read TV after 30 min.						
Turbidity value (TV)	15	15	20	10	25	7
Corrected Value	0		+10		+18	

0.2 mg. st'd. TV = 40
0.1 mg. st'd. TV = 19
Therefore, 0.1 ml. 1:18 dilution contains 1 TRU or 180 TRU/ml.

supernatant was removed, the residue resuspended in a few ml. *p*H 6.0 acetate buffer, and centrifuged again. The two supernatants were combined and the *p*H of the extract was brought to 4.2 (glass electrode) using 2N acetic acid. The acidified mixture was then placed in the ice box for an hour to permit the settling out of an inactive, flocculent precipitate. The solution was then centrifuged, the supernatant removed, and the *p*H of the supernatant readjusted to *p*H 7.0 with 2N NaOH. This neutral solution of the enzyme was centrifuged to remove a small amount of inactive precipitate. The volume of the clear, slightly pink extract was adjusted so that 1 ml. of solution was equivalent to 1 gm. of tissue.

Duplicate assays were made on the testes of adult normal rats, of rats hypophysectomized for 30 days, on rat salivary glands, and a single assay on chicken testes. The results are presented in Table 2.

Hyaluronidase assays of semen in relation to sperm count

A preliminary study was made on the applicability of the turbidimetric method to the assay of enzyme concentration in semen of

bulls and men as related to sperm count. The bull semen was collected and sperm counts made by Dr. Glenn Salisbury. The human samples were collected in New York and the sperm counted before toluene was added.

The results of assays on several semen samples from two bulls are given in Table 3. It was noted that in general the greater the concentration of sperm the greater was the amount of hyaluronidase pres-

TABLE 2

Tissue	Number of animals per assay	Activity
Normal rat testes	4	10 TRU/gm.
Hyp. rat testes	4	less than 2 TRU/gm.
Chick testes	1	0
Rat salivary glands	2	0

TABLE 3. HYALURONIDASE ASSAY OF BULL SEMEN

Bull	Sperm (million per ml.)	TRU/ml.	Hyaluronidase Index TRU/million sperm per ml.
AJ	1500	1600	1.1
	1400	1500	1.0
	1050	1200	1.1
	850	900	1.1
	600	800	1.3
REG	1400	2800	2.0
	1250	1200	0.9
	950	1600	1.7
	700	1200	1.3
	153	200	1.7

ent. In assays on other bulls the same trend prevailed. The absolute amount of enzyme however in relation to sperm count varied with the individuals. This individual variation in the relation of sperm number to enzyme concentration was best expressed by the *hyaluronidase index*, i.e., TRU/ the number of millions of sperm per ml.

Studies on samples of human semen showed tremendous variations in enzyme concentration in relation to the sperm count (Table 4), so that correlation was not possible. An aspermic sample failed to reveal any enzyme, however, and the highest sperm count gave the highest concentration of enzyme.

DISCUSSION

The results of these experiments show that the turbidimetric method of assaying hyaluronidase is readily applicable to semen and tissue extracts. The method is relatively simple, rapid, and only requires apparatus that is usually found in a laboratory.

In studying problems of fertility in relation to hyaluronidase it may be desirable at certain times, and when possible, to make a bio-

logical assay using the rat-ova test (Leonard and Kurzrok 1946). For routine analysis of the enzyme in semen samples the use of the rat-ova test is not practical for obvious reasons. It was possible to relate quantitatively a given enzyme preparation in terms of *rat-ova units* or *turbidity-reducing units*. With the preparation of hyaluronidase used, it was found that 1 rat-ova unit and the TRU under these conditions were the same (.033 mg.).

By this method of determining hyaluronidase in tissue it was possible to show that the rat testes must contain sperm to have a max-

TABLE 4. HYALURONIDASE ASSAY OF HUMAN SEMEN

Case	Count (million/ml.)	TRU/ml.	Abnormal %	Motility %
485	180	150	30	85
491	165	25	60	40
P501	152	12	55	50
P517	150	11	25	70
MA-7-17	140	9	10	85
493	129	80	10	90
496	110	16	50	75
494	106	80	30	70
P516	105	40	35	50
P500	100	60	20	90
483	76	50	60	50
497	52	16	25	70
484	51	75	65	45
AI-C(7-29)	48	16	25	85
495	43	20	25	80
498	25	16	60	10
486	9	25	80	3
P515	aspermia	0	0	0

imal amount of the enzyme. The testes of the hypophysectomized rats contained less than 2 TRU of the enzyme per gram whereas the normal testes contained 10 TRU per gram. Similar results were obtained with the rat-ova test. Since the hypophysectomized rat testes possess spermatogonia, it is quite possible that what enzyme was present came from these cells. Salivary gland of the rat and fowl testes contained no demonstrable amount of the enzyme. Similar results were reported by Duran-Reynals (1942) using the "spreading factor" test for hyaluronidase.

Further evidence that sperm produce the enzyme was shown in the analysis of bull semen. Several samples of semen from a single bull indicated a closer relationship of sperm count to enzyme concentration than did samples from different bulls. A large amount of data is being accumulated on bull semen in relation to hyaluronidase in conjunction with the work of Dr. Glenn Salisbury on artificial breeding and will be reported at another time.

In the small series of tests made on human semen samples a direct correlation between sperm count and enzyme concentration appeared lacking. Kurzrok, Leonard, and Conrad (1946) have reported a sim-

ilar study on a larger number of cases but used a different test method and they found that the correlation held only within certain limits of sperm concentration. Others, however, have stated that a definite correlation exists (Joel and Eichenberger, 1945; Werthessen et al., 1945). It appears from the results of the present study that certain qualifications attend the interpretation of these correlations. The bull semen was obtained from uniformly normal, healthy, well bred animals which were being used regularly for breeding purposes yet even here variations between bulls was noticeable. The human samples were taken singly from a heterogeneous population and were brought to the physician because of marital sterility. The relationship between sperm numbers and enzyme concentration awaits further study in view of these results.

SUMMARY

The turbidimetric method of Kass and Seastone for determining hyaluronidase was adapted for assaying the enzyme in semen and tissue. Activity of the enzyme is expressed in *turbidity-reducing units.* Differences in properties of bull and human semen necessitated modifications in preparing the material for assay. Preliminary studies on bull semen showed that a correlation between sperm numbers and hyaluronidase concentration could be made only when samples were assayed and compared from one individual. Assays of single samples from human semen are reported and indicate wide variation in the relationship between sperm count and enzyme concentration. The results of tissue analysis showed that, on a gram basis, normal rat testes contained 10 TRU, hypophysectomized rat testes <2 TRU, chicken testes and rat salivary glands no activity.

ACKNOWLEDGMENT

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NOTES AND COMMENTS

PREPARATION OF RELAXIN FROM SOW CORPORA LUTEA RESIDUE[1]

The purpose of this note is to describe a method of preparation of a water-soluble substance, relaxin, from a residue of sow corpora lutea.

MATERIALS AND METHODS

Fresh frozen sow corpora lutea were used as the original source of relaxin. This tissue was extracted with methanol for its estrogen and progestin content, and the tissue residue pressed into pellet form. We are obligated to Dr. G. F. Cartland, of the Upjohn Company, Kalamazoo, Michigan, for

TABLE I. FLOW SHEET OF STEPS OF MAIN FRACTIONATIONS

Steps in procedure	Total wt. gm.	Total units $\times 10^3$	Units/ gm.	Units/ mg. N	% Yield	Purification
1. Fresh Frozen Sow C. L.	128,000 (wet)	?	—	—	—	—
2. Methanol Insol. Res. (Pellets)	40,000	128	3	—	—	1
3. Acid Extract as SAS ppt.	130	128	900	7	100	300
4. pH 0.75 Extract	51	128	2500	23	100	833
4a. pH 3.61 Extract	45	126	2800	28	98	933
5. 75% SAS ppt. as pH 7.5 Extract	28	126	4500	34	98	1500
6. 0.33–0.66 SAS Fraction	15.2	96	6300	54	75	2100
7. 0.47–0.85 ETOH Fraction	4.3	68	16,000	140	53	5330

this tissue,[2] and for working up a few batches of this waste residue through step 3 (Table 1). The method of assay of relaxin and the method for its preliminary extraction and concentration from the pellets were the same as previously described (Abramowitz et. al., 1942, 1944). Dry weights, ash and nitrogen were determined in the usual manner.

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[1] Aided in part by a grant from the Milton Fund to Professor F. L. Hisaw.
[2] A comparison between the relaxin content of fresh corpora lutea and the relaxin content of this residue is not possible since this residue had been dried and stored for a long period of time before it was extracted. Consequently, as shown in the flow sheet, the purification was calculated on basis of the hormone content present in this residue at the time of extraction.

RESULTS

The extraction procedure, briefly summarized, consists of (Step 1) extracting fresh frozen corpora lutea with methanol to obtain estrogens and progestin, (Step 2) the preparation of the tissue residue from Step 1 in the form of semi-dry pellets, and (Step 3) extraction of relaxin from the pellets by 2% HCl in the presence of 1 M. NaCl, and precipitation of the hormone by ammonium sulfate. From 40 kg. pellets, a precipitate containing 130 grams solids was obtained.

Step 4

The precipitate from Step 3 was dialyzed against cold, running tap water until salt free, and then against distilled water at room temperature. The dialyzed preparation was a brownish, turbid emulsion, containing between 10 and 30 gram equivalents (gm. eq.) of fresh tissue per ml. Concentrated HCl was added drop by drop with stirring until the pH of the emulsion was 0.75. The emulsion immediately broke into a brown precipitate, which was removed by centrifuging, and a clear brown solution containing the hormone.

Step 4a

Twenty gm. NaCl was added for every liter of the pH 0.75 extract, and the pH adjusted to 3.61 with 10% NaOH. Precipitation of a small amount of fine, almost gelatinous material occurred over the range of pH from 3.4–3.8, and turbidity was maximal at pH 3.61. The precipitate was removed by centrifuging, and washed thrice with 1.28 l. portions of 2% NaCl (10 ml./kg. eq.) and discarded. The washings and main supernatant solution were brought to pH 7.0 with 1% NaOH.

Step 5

The relaxin was now concentrated by pouring the solution at pH 7.0 with gentle stirring into 3 volumes of saturated Ammonium Sulfate Solution (SAS). After standing at room temperature for 2 days, the material was filtered by gravity. The 75% SAS precipitate was emulsified into a paste with 1.28 l. distilled water and dialyzed. The dialyzed solution was adjusted to pH 7.5 with 1% NaOH and stirred for 2 hours. Insoluble material was centrifuged off, washed twice at pH 7.5 with distilled water in amounts of 2 ml./kg. eq. and rejected. The combined supernatant solution had a concentration of between 50 and 100 gm. eq. per ml., the variation being due to the amount of salt present in the 75% SAS precipitate.

Step 6

To the pH 7.5 extract, one-half volume of SAS added dropwise with stirring, and the mixture allowed to stand in the cold (5°C.) for 3 days. A fluffy precipitate rose to the top and was discarded after being washed twice with small amounts of 33% SAS (2 ml./kg. eq.). The 33% SAS extract was brought to 66% by the addition of an equal volume of SAS and allowed to stand in the cold for 2 days. The precipitate was removed by centrifuging and dissolved in 1.28 l. of water, and refractionated with 33% and 66% SAS as above. The final 66% SAS precipitate was suspended in 256 ml. of water and dialyzed, as in Step 4. The dialyzed solution was brought to pH 7.3, insoluble material removed and washed with water as before, and the combined solution diluted to 1.28 l.

Step 7

Sodium chloride (12.8 gm.) was added to the solution, chilled to 5°C. One volume of 95% ethanol at 5°C. was added drop by drop with stirring (at a rate of about 3 ml./minute). The solution was kept in the cold overnight, and then centrifuged. The 47% extract was stored. The precipitate was dissolved in 640 ml. cold 1% NaCl and reprecipitated by the addition of one volume of 95% alcohol. This procedure was again repeated. All three 47% alcohol extracts were combined, and additional cold 95% ethanol added to a total alcohol concentration of 85%. After standing overnight, the precipitate was collected and dissolved in 640 ml. of 1% NaCl solution and the entire procedure was repeated.

Step 8

The 85% ethanol precipitate was collected and suspended in 250 ml. water, and dialyzed against M/15 phosphate buffer at pH 7.6. The dialyzed solution when centrifuged free of insoluble material, can be prepared as a white powder by drying with 4 volumes of cold acetone. The activity of the powder varied with different batches but was usually 20–30 units/mg.

SUMMARY

A method is described for the preparation of relaxin from sow corpora lutea residue, as a stable, water-soluble powder with an activity of 20–30 units/mg. and a N content of 11%. The activity[3] of this powder is about the same as that reported by Fevold et al. (1930), but it can be achieved with simpler reagents and with fewer manipulations.

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INCREASED PITUITARY WEIGHT PRODUCED BY OESTRONE IN INTACT AND CASTRATED RATS[1]

Smith and Smith (1944) have claimed that the pituitary glands of castrated male rats increase in weight after the injection during 5 days of a total dose of 10 μg. of oestrone, while 500 μg. are required to produce the same effects in intact rats. During an investigation of the quantitative rela-

[3] The units used are considered to be roughly equal.

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[1] This work was aided by an apparatus grant from the Central Research Fund, University of London.

tions between gonadal and hypophyseal secretions we repeated this experiment, but could not confirm Smith and Smith's finding. The matter is important as the difference in response is used to support the view (Smith, 1944) that it is not oestrone itself that influences pituitary secretion, but a metabolite of oestrone with little oestrogenic activity.

METHODS

Animals were all adult males of the albino Wistar strain bred in this Institute.

Injections were all given twice daily for 5 days in oily solution. Total

TABLE 1. EFFECTS IN ADULT RATS (98–195 GM.)

(5 rats per group)

Intact rats		Castrated rats	
Total dose of oestrone (μg./100 gm.)	Pituitary weight (mg./kg. $\pm \sigma m$.)	Total dose of oestrone (μg./100 gm.)	Pituitary weight (mg./kg. $\pm \sigma m$.)
0	27 ± 2	0	28 ± 3
100	26 ± 1	5	24 ± 3
250	33 ± 1	25	28 ± 3

TABLE 2. EFFECTS IN ADULT RATS (100–120 GM.)

(5 rats per group)

	(Pituitary weight (mg./kg. $\pm \sigma m$)			
	Intact rats		Castrated rats	
Total dose of oestrone μg./100 gm.)	Control	Injected	Control	Injected
10	33 ± 2	32 ± 1	30 ± 2	34 ± 2
25	30 ± 2	35 ± 2	35 ± 2	36 ± 2
50	34 ± 3	40 ± 2	39 ± 1	44 ± 2
100	32 ± 2	41 ± 1	33 ± 3	44 ± 3
250	37 ± 1	51 ± 2	34 ± 1	60 ± 4

doses are in all cases expressed as μg. per 100 gm. body weight and were given in volumes of 0.2 ml. per 100 gm. per injection.

Body weights were recorded daily to allow adjustment of the dosage.

Castration was performed 24 or 48 hr. before the first injection.

Organ weights were recorded after the organs had been fixed in Bouin's fluid and transferred to 70% alcohol, the animals having been killed 16 hr. after the last injection. Organ weights were found to be much less variable when expressed as proportions of body weight and are given in this form in the tables.

RESULTS

Effects in adult rats

Using the doses of oestrone found effective by Smith (1944) we were unable to show even a 10-fold difference in sensitivity between intact and castrated rats. The doses used in the castrated rats had no effect, while only the highest dose increased the pituitary weight in the intact rats (Table 1).

Effects in adult rats of uniform weight

We then determined the threshold doses of oestrone needed to increase pituitary weight in rats all weighing 100–120 gm. (the weights in the previous experiment had been 98–195 gm.). This selection meant that fewer rats were available so that only one dose could be tested at a time; controls were run concurrently in all cases. The results in these generally lighter animals (Table 2) showed that 100 μg. total doses produced significant increases in pituitary weight in intact and castrated rats but that lower doses did not. There were no statistically significant differences between the organ weights of any two injected groups given the same dose. We conclude that there is no difference in the sensitivity of the two groups.

Effect in litter mates

When the method of Smith and Smith was exactly repeated by using litter mates and injections in aqueous alkaline solution, there was greater variation in the weights recorded but still no evidence of greater sensitivity in the castrated animals.

SUMMARY

No differences could be detected in the increases in putuitary weight produced by oestrone injections in intact and castrated adult male rats.

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THE EFFECT OF THYROID ADMINISTRATION ON THE THYROTROPIC ACTIVITY OF THE RAT PITUITARY

Reforzo-Membrives (1943) claimed to demonstrate a thyroid inhibiting action in the pituitaries of rats treated with desiccated thyroid. Although this result has been widely quoted there has been no confirmation reported. It seems desirable to report the results of our own investigations which failed to confirm the presence of thyroid inhibiting action in the pituitaries of rats treated with desiccated thyroid.

METHODS

The rats used were of a Wistar strain, and were about 14 weeks old at the commencement of the experiment. For the assays male and female rats were used in equal numbers. They were fed on diet S2 previously described

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(Griesbach and Purves, 1943a). The thyroid treated rats received 100 mgs. daily of thyroid powder containing 0.2% of iodine (U.S.P. standard). The thyroid powder was mixed with the rats' food, and was given for 14 to 18 days before the rats were killed.

The guinea-pigs used for the assays were three weeks old. For the assay of pituitaries of thyroid treated rats, litter mate controls of the same sex as the injected mate were used. The injected guinea-pigs received a saline suspension of fresh rats' pituitaries equivalent to one-half of a rats' pituitary daily. These injections were given on five successive days and the guinea-pigs killed on the sixth day. The thyroids were dissected out and weighed. The subsequent histological examination and measurement of cell height were carried out as previously described (Griesbach and Purves, 1943b). An assay of normal rats' pituitaries was carried out separately.

TABLE 1. ASSAY OF THYROTROPIC ACTIVITY OF HYPERTHYROID RAT PITUITARIES MEASUREMENT OF GUINEA PIG THYROIDS

Thyroid weight mgs./100g.			Acinar cell height μ		
Litter No.	Control	injected	A Control	B injected	B − A
1	13.9	12.1	6.4	6.1	−0.3
2	15.6	14.4	7.0	7.4	+0.4
3	9.8	11.2	6.5	6.4	−0.1
4	11.2	11.0	5.8	5.9	+0.1
5	17.7	17.5	9.8	9.6	−0.2
6	21.7	25.0	9.5	9.9	+0.4
7	16.7	21.2	9.4	10.2	+0.8
8	27.9	21.3	9.8	10.0	+0.2
Mean	16.8 mg. %	16.7 mg. %	8.03μ	8.19μ	+0.16μ SE ±0.14μ

TABLE 2. ASSAY OF THYROTROPIC ACTIVITY OF NORMAL RAT PITUITARIES. 8 GUINEA PIGS IN EACH GROUP

Thyroid weight mgs./100 g.			Acinar cell height μ		
Control	Injected	Increase	Control	Injected	Increase
11.54 ± 0.35	32.80 ± 2.44	21.26 ± 2.46	6.67 ± 0.19	12.91 ± 0.20	6.24 ± 0.28

RESULTS

The results of the assay of thyroid-treated rats' pituitaries are reproduced in Table 1. It will be seen that the injections of rat pituitaries were without effect on the guinea pig thyroids. The slightly higher average cell height for the pituitary treated group (8.19μ; control 8.03μ) is not statistically significant.

The results of the assay of normal rats' pituitaries are summarized in Table 2. The injection of two and a half normal pituitaries per guinea pig increased the weight of the guinea pig thyroids to nearly three times that of the controls while the cell height was almost doubled. The actual weight increase was 21.26 mgs. and the cell height increase was 6.24μ, both these effects being highly significant.

DISCUSSION

The results here reported show that thyroid administration greatly reduces the thyrotropic hormone content of the rat pituitary. The hormone content after thyroid administration is certainly less than five per cent of the normal, indeed it is doubtful whether any thyrotropic activity is detectable in these pituitaries. However there is clearly no thyroid inhibiting effect of the kind postulated by Reforzo-Membrives. The differences between our experiment and that of Reforzo-Membrives are that in our experiment the thyroid treatment was given for 14 to 18 days as compared with 22 to 32 days, and two and a half pituitaries were injected over five days as compared with four pituitaries injected over two days. If a thyroid inhibiting action of the pituitary is of physiological significance in conditions of hyperthyroidism it might be expected to reveal itself equally as well under our conditions as under those of Reforzo-Membrives. The data given by Reforzo-Membrives show that the treated guinea-pigs had after two days' treatment thyroids 26% less in weight and cell heights 1.6μ less than the controls. Our experience of the relation between cell height response and weight response in the guinea-pig thyroid suggests that this weight change is out of proportion to the small change in cell height. We have found (Griesbach and Purves, 1943b) that when the cell height is small the effect of variations in cell size on thyroid weight is neutralized by opposing variations in the amount of colloid storage so that the weight is quite insensitive to variations of cell activity until the cell height exceeds 8μ. Our own data show that great variations in thyroid weight may occur in young guinea-pigs and that low thyroid weights are associated with low epithelium heights, as will be seen by comparing litters 3 and 4 in Table 1 with litters 7 and 8. These litters are arranged in chronological order of birth, the last litter being born only about four weeks after the first. Had our experiment been conducted in a less rigorous manner and the first four litters been given pituitary injections while the last four were retained for control an apparently significant but quite erroneous indication of a thyroid inhibiting action would have been observed.

Reference should be made to the results of basal metabolism measurements on pituitary injected guinea-pigs reported by Reforzo-Membrives. Some of these results, in particular those of guinea pig No. 2 in Reforzo-Membrives' Fig. 6, are not consistent with the hypothesis that these pituitaries depress metabolism by inhibiting thyroid activity since the metabolism is depressed by as much as 48% after only two days of pituitary treatment. Even total thyroid ablation does not affect metabolism with this suddenness, or reduce it to such low levels.

Adams and Jensen (1944) have reported a careful study of the effect of large thyroxine dosage on the thyrotropin content of the mouse pituitary. Their result, that thyroid treatment greatly diminishes thyrotropic activity, is in accord with ours, but the failure to demonstrate in their experiments a thyroid inhibiting action is open to the possible objection that since the cell height of the control chicks which they used as test animals was only 2.15μ, the thyroids may have been maximally involuted and therefore incapable of responding to a thyroid inhibiting agent. This is not the case in our experiments since our guinea-pigs normally show a high degree of thyroid activity as the cell height measurements demonstrate.

SUMMARY

Rats were given 100 mgs. daily of desiccated thyroid (iodine content 0.2%) for 14 to 18 days. Their pituitaries were assayed for thyrotropic effect on the guinea pig thyroid, two and a half pituitaries being injected into each guinea pig over a period of five days. These pituitaries were without significant effect on the weight or histology of the thyroids of the injected guinea pigs. From a comparison with the results of the assay of normal rat pituitaries which increased the weight of the guinea pig thyroids by 21.26 mgs. and the acinar cell height by 6.24μ, it is concluded that thyroid administration depressed the thyrotropic activity of the rat pituitary to less than five per cent of the normal level. However there was no evidence of the presence of the thyroid inhibiting activity described by Reforzo-Membrives in the pituitaries of rats treated with desiccated thyroid.

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NEW BOOK

CARBOHYDRATE METABOLISM. By Samuel Soskin, M.D. and Rachmiel Levine, M.D., University of Chicago Press, 1946, 305 pages (75 figures, 42 tables). Price $6.00.

There is a need for texts on special scientific fields of restricted scope which are less abstruse and detailed than the usual technical monograph. Such volumes (too rare) can serve both the student and a wider public of investigators and practitioners who may wish to review the fundamentals and recent significant advances in fields allied to their own. The authors of the present volume have attempted to fulfill this need on the subject of carbohydrate metabolism. In the main, they have succeeded, although some of the material can be recommended to the non-specialist only with certain reservations.

The material in the book is presented in five sections: I. The Biochemistry and Energetics of Carbohydrate Metabolism; II. Introductory Physiological Considerations; III. Critical Survey of the Classical Criteria of Diabetes; IV. The Role of the Endocrine Glands in Carbohydrate Metabolism; V. Integration of Physiological and Clinical Aspects. The first section contains an excellent outline of intermediary carbohydrate metabolism, including a clear elementary exposition of the important role of phosphate bond energy in these processes. Part II comprises short chapters on the nature and occurrence in tissues of materials important to carbohydrate metabolism, on the site of origin of the blood sugar, and on the use of diabetic animals in the study of carbohydrate metabolism. In part III are chapters on the D:N ratio, ketosis, the R. Q., gluconeogenesis from protein and from fat, and on the utilization of carbohydrate in the whole organism. A critical discussion of the older literature on these subjects is included. The roles of the hormones from the pancreas, adrenal cortex, thyroid and anterior pituitary are discussed in part IV; and in part V principal attention is paid to the homeostatic regulation of the blood sugar, and to the pathological physiology of carbohydrate metabolism and its clinical application.

The organization of the material is good, the fundamental observations are lucidly described, and the views of the authors are stated unequivocally. Unfortunately, devotion to the either-or type of argument has led the authors to overlook a number of possibilities in interpretation of the data at certain points, and sometimes to take the far-reaching conclusions arrived at in the course of such arguments as the factual bases of further hypotheses. The authors' salutary discussion of the uncritical use of calculations based on the R. Q. and the D:N ratio is a reminder that interpretations of even the most fundamental experiments may undergo revision in time. A large number of figures, some using pictorial analogies, others smooth curves or graphs of average values, illustrate the authors' conceptions. In the tables, the data are often presented in terms of percentages—a practice helpful to quick comprehension by those not used to figures, although occasionally misleading

to author and reader alike. Generous and well-selected lists of references, including titles, are appended to each chapter. These, together with the figures and tables make up over one-fourth of the book. In the text, constant emphasis is placed upon the integration of individual processes of carbohydrate metabolism in a larger homeostatic mechanism; and frequent reference is made to the interlocking relationships between carbohydrate, fat, and protein metabolism.

Greater emphasis might have been placed upon the evidence for the occurrence and for the logistic importance of fat formation from carbohydrate. As the authors point out, a large part of the usual dietary consists of carbohydrate, yet the stores in the tissues at any time are relatively quite small. However, they deal only briefly with the older general literature, and omit the newer work on the relative rates of turn-over of carbohydrate and of fat which indicates that fat formation is an important pathway of carbohydrate utilization. A theoretical equation is given for fat synthesis from glucose, without any discussion of why this one, of numerous possible ones, was chosen. To be sure, we know little of the details of this process, but similar lack of knowledge has not prevented the authors from presenting, in text and diagram, schemes for the reverse conversion. Some other omissions may perhaps be due to a delay in publication date, since there are few references more recent than 1943.

The views of the authors on the nature of diabetes, developed mainly in part III, are well known to those who are familiar with the work of the senior author or with his review on the regulation of the blood sugar, from which considerable sections of the present book are taken. These tenets, which form the basis of the discussion in later portions of the book, are not entirely those held by the majority of investigators in this field. Most of the experimental data presented were taken, in diagram form, from papers by the authors. The reader is not always warned when he is on controversial ground: certain evidence which may be interpreted to oppose the views of the authors has been either omitted, disregarded, or dismissed with irrelevant argument, and in a few instances supporting material has been accepted uncritically. In consequence, the book has been removed to this extent from the status of an auxiliary text to that of a monograph which must be read with some knowledge of the material discussed in it.

In the section on endocrine regulation of carbohydrate metabolism, as in the rest of the book, a comprehensive review has not been attempted. The majority of the data considered by the authors have been derived from observations of the dog, and where the conclusions to be drawn from this work are at variance with evidence obtained in smaller animals, the authors have not hesitated to favor the former. Indeed, in an earlier chapter it is remarked that "the subject has been complicated by the use by various investigators of other animals than the dog." The authors appear not to distinguish any differences between the e ects of adrenalectomy and of hypophysectomy on the metabolism of carbohydrate, and any effects of either hormone on the utilization of carbohydrate is vigorously denied. That adrenalectomy affects primarily the rate of oxidation of carbohydrate has, in fact, been suggested by only a few investigators, *not* those who are quoted in the book as supporting this view. The impression is left that the thyroid

has a role similar to that of the pituitary and adrenal, differing only quantitatively in its expression.

Few mechanical errors have been remarked. In Fig. 7, the structure of flavin adenine dinucleotide is shown containing two ribityl groups, both called ribose, whereas the flavin nucleotide contains the ribityl group and the adenosine portion a riboside linkage. The phosphorolysis of glycogen depicted in Fig. 12 should show the reaction with a terminal glucose unit, rather than an intermediate linkage. Figure 14 omits a hydrogen atom from C-5 of fructose. Weinhouse, et al. (p. 119) used "heavy carbon," C^{13}, rather than radioactive isotope as stated, while Hastings, et al. (p. 144) used radioactive compounds instead of "heavy" carbon.

The excellent design and printing of this book increase the pleasure of reading it.

JANE A. RUSSELL

NOTICE

The American Academy of Allergy will hold its annual convention at Hotel Pennsylvania, New York City, November 25–27 inclusive. All physicians interested in allergic problems are cordially invited to attend the sessions as guests of the Academy without payment of registration fee. The program has been arranged to cover a wide variety of conditions where allergic factors may be important. Papers will be presented dealing with the latest methods of diagnosis and treatment as well as the results of investigation and research. Advance copies of the program may be obtained by writing to the Chairman on Arrangements, Dr. Horace S. Baldwin, 136 East 64th Street, New York City, prior to November 10th.